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BIOMONITORING OF SURFACE WATER BY SYNCHRONOUS CULTURE OF *CHLORELLA VULGARIS* ALGAE

A possible use of synchronous culture of *Chlorella vulgaris* algae has been examined as a short-term bioassay for water quality control. Experiments were performed with algae cultivated in liquid media prepared with distilled water (control) and with water aliquots sampled from the Goczałkowice Reservoir. It has been demonstrated that changes in the absorbance (at 680 nm) of algae liquid synchronous culture and the rate of algae cellular division may be useful as criteria for water quality control. Changes in the algae metabolic activity are clearly a sign of the July flood and autumnal water quality changes.

1. INTRODUCTION

More than one hundred of short-term bioassays are now available to detect toxicity, mutagenicity and carcinogenicity of chemicals [1]. Results of these bioassays are verified through reactions of given organisms with particular contaminants.

Biotests, besides their widely accepted use for standard investigation of various physical and chemical properties, are employed for water quality monitoring in the US, Canada and the European Union [2, 3]. Bioindicator organisms, specifically responding to water contaminants, are applied in such biotests. A biotest itself cannot answer a question of which of many contaminants is responsible for change of the biological activity of a chosen bioindicator [1]. Moreover, interactions between substances present in water also influence the biological activity of bioindicators and – in consequence – the toxic effect on the organism used as a bioindicator as its response to water pollution. Consequently it is impossible to evaluate toxic effects in living environments using only standard physicochemical methods.

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Many organisms such as microalgae *Pseudokirchneriella subcapitata* (test Algaltoxkit F), rotifer *Brachionus calyciflorus* (test RotoxkitF), protozoans *Tetrahymena pyriformis* and *T. thermophila* (test Protoxkit F), luminescent bacteria *Vibrio fischeri* [4], crustaceans *Daphnia magna*, *D. pulex*, *Ceriodaphnia dubia*, *Thamnocephalus platyurus* (tests Daphtoxkit F magna, Daphtoxkit F pulex, Ceriodaphtoxkit F, Thamnotoxkit F) and fish *Pimephales promelas* [5, 6] are used as bioindicators.

Chlorella algae, commonly found in natural environment, are also used as a water quality bioindicator. *Chlorella vulgaris* asynchronous cultures, in which algae cells are at various life stages, are proposed to be used as a bioindicator in biotests [2]. It has been shown in many studies [7, 8] that synchronous cultures of *Chlorella vulgaris* algae in which the cells are at the same stage of life were useful to explain how the cells at individual life stages were affected by the tested substances. Consequently the synchronous cultures provide more uniform responses for changes in toxicity as compared with asynchronous cultures.

Wilczok et al. [9] used synchronous cultures of *Chlorella vulgaris* algae in environmental investigations performed in 1984. The aim of that study was to evaluate the toxicity of wastewater discharged from industrial plants located in the Odra River basin. Changes of the culture absorbance and the rate of cellular division in a synchronous culture of *Chlorella vulgaris* algae in relation to a control culture were used in the experiments as a toxicity criterion. A synchronous culture of *Chlorella vulgaris* algae was also applied to investigate the toxicity of wastewater from the "Skotan" tannery located in Skoczów [10].

Results of spectrophotometric and chromatographic (by means of high performance liquid chromatography) analyses of photosynthetic pigments extracted from daughter cells, cultured in a single life cycle of a synchronous *Chlorella vulgaris* culture in the presence of tested water may be considered as a water quality criterion [11].

However, these relatively time-consuming and expensive investigation methods can be replaced by monitoring of changes of absorbance of the algae culture during the first 10 hours of the life cycle and the rate of cellular division per life cycle. The aim of this study was to examine these parameters in the *Chlorella vulgaris* synchronous culture, in respect of their use as a quick and inexpensive method of water quality control.

2. MATERIALS AND METHODS

Description of the study area. The Goczałkowice water reservoir was built to supply drinking and industrial water to the region of Upper Silesia as well as to protect the valley of the Vistula River from floods. Water samples used for culturing of *Chlorella vulgaris* algae were collected once a month (June–October, 1997) at seven sampling points situated in the area of the Goczałkowice Reservoir (Fig. 1). The samples

were placed into hermetically closed polythene containers. Water was filtered through the membrane filters (Millipore, 0.45 µm pore diameter) before experiments.

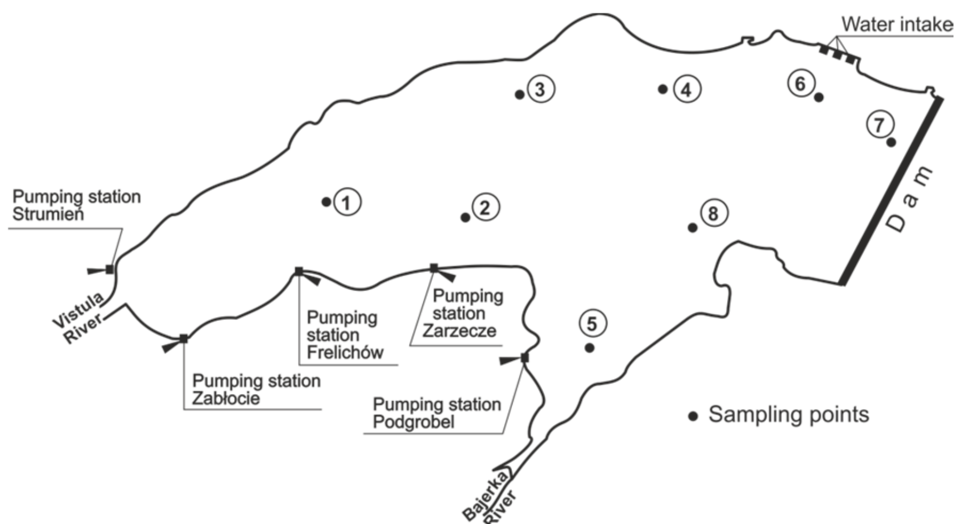

Fig. 1. Goczałkowice Reservoir with the location of water sampling points

Bacteriological testing and evaluation of the physicochemical properties of water were conducted by the Upper Silesian Water Supply Company. The concentration of metals in investigated water was determined using the atomic absorption spectrometer (AAS-3 Carl Zeiss, Jena) in an air-acetylene flame. Analyses were made at the Department of Instrumental Analysis, Medical University of Silesia.

Characteristics of algae and conditions of the culture. Synchronic culture of *Chlorella vulgaris* algae, Beijerinck 1890, strain A-8 were used in the experiments. Algae were cultured in the sterile standard mineral Kühl and Lorenzen liquid medium [12] modified by Borns et al. [13]. Investigations were conducted in culture liquid media prepared with distilled water (control) and water sampled at the sampling points of the Goczałkowice Reservoir (testing water) (Fig. 1). Control culture of *Chlorella* was conducted every month. Individual algae culture of each water sample was performed under stable conditions (temperature 30 °C, illumination 15 000 lux on the reactor surface) during 10 h of the light period followed by 14 h of the dark period. During the light period, the cultures were aerated (30 $dm^3 \cdot h^{-1}$) with air supplemented with 2% CO_2 while during the dark period just by air only by the Wilczok and Mazurek [14] method. The moment of turning on the light (t = 0 h) was established as a start time of life cycle of *Chlorella vulgaris* algae. One culture cycle of *Chlorella vulgaris* algae population complies with a life cycle of a single cell in a synchronic system. The number of cells accounted for $5\times10^6\pm0.5\times10^6$ cells per 1 cm^3 of medium, once the

light was turned on. The algae cells grew synchronously from aplanospores to mother cells during the 10 hours of the light phase, and the division of cells took place at the beginning of the dark phase (Fig. 2) [15].

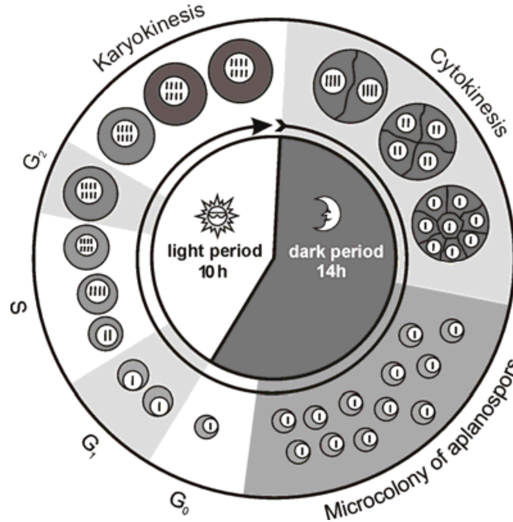

Fig. 2. Scheme of the cell life cycle
of *Chlorella vulgaris*, Beijerinck 1890 [15]

Estimation of the biomass increase. The samples of algae suspension were collected from each of the culture reactors at preset hours (0 h, 5 h, 10 h, 24 h) of the algae life cycle, and the cells were counted in a Bürker's chamber (magnification 400×) using the Doccuval light microscope (Carl Zeiss, Jena). As a result, the cells number in 1 cm^3 of the algae culture has been determined [10]. The criterion of the biomass increase was the coefficient of the cells division (K_d) in the investigated cultivation system. This coefficient makes possible to estimate the quantity of aplanospores released from one mother cell in a definite life cycle. K_d has been calculated using the equation:

$$K_d = \frac{n_{24}}{n_0}$$

where: n_{24} – arithmetical mean of the cell number in 1 cm^3 of culture suspension sampled in 24th hour of the algae life cycle, n_0 – arithmetical mean of cell number in 1 cm^3 of culture suspension, sampled at the beginning of the algae life cycle.

For the unequivocal interpretation of K_d changes observed during various experiments, the relative coefficients of the cells division (K_{dr}) have been calculated as the

quotients of the coefficients estimated for the algae cultures growing in the medium prepared from the tested water and the control algae culture.

Spectrophotometric study of metabolic activity of cells. The differences between absorbances of the culture measured by a Hewlett Packard 8452A spectrophotometer at the wavelength of 680 nm at successive hours of the algae life cycle light phase (0–10 h) were used as *Chlorella vulgaris* cells biological activity measures. The absorbances were referred to 10^6 cells in 1 cm^3 of the liquid culture.

Statistical analyses. The one-way ANOVA with post hoc tests were used to test the statistical significance of the differences among the obtained arithmetical means of relative coefficients of the cells division calculated for each month.

The analyses of covariance (ANCOVA) with tests of homogeneity of the linear regression coefficients were used to estimate the dynamics of changes in the metabolic activity of cells in investigated liquid cultures. It was taken into consideration that the dynamics of changes in the absorbance values recalculated per 10^6 algae cells may be described by the equation:

$$\left(\frac{\text{absorbance}}{10^6}\right)^{1/2} = at + b$$

where: t is the time of the algae culturing, a – the regression coefficient, b – the shift coefficient. Analyses have been carried out with the significance level $p < 0.05$. Statistical analyses have been performed using the Statistica v.5.1 software.

3. RESULTS AND DISCUSSION

Water environments are contaminated by over million different pollutants originating from natural and industrial sources. Their analyses by chemical methods are expensive and complicated due to variety of interactions between them. These interactions lead to a complex influence of pollutants on living organisms. Biotests make possible to evaluate the total influence of polluted environment on bioindicators but they do not show specific chemical compounds responsible for changes in their bioactivity [1]. Mixture of pollutants present in water affect indicatory organisms and the net effects may be quite different from those observed for individual substances due to antagonistic or synergistic interactions between them. As an example, manganese, zinc, and ammonium salts cause decrease in the cadmium toxicity towards *Chlorella fusca* [16]. On the other hand, copper ions show synergistic effect on the cadmium toxicity [7], and act antagonistically with calcium and chromium(VI) ions [17]. Historically, biotests were based on measuring the toxicity of individual chemical com-

pounds. Thus, their application was limited to a potential hazardous impact of various simple pollutants [1]. For example, it has been shown that toxicities of metal ions towards *Chlorella vulgaris* algae may be arranged in the following descending order: Hg > Cu > Cd > Fe > Cr > Zn > Ni > Co > Mn [18]. Apart from metal ions, many organic substances are also toxic to *Chlorella vulgaris* algae. It has been indicated that benzo(a)pyrene may penetrate and accumulate in *Chlorella vulgaris* cells [19]. It must be taken into account that the organisms are rarely exposed to single pollutants and the exposure to the pollutants' mixture is more common in a natural environment. To evaluate water trophic state, Bednarz [20] performed a bioassay by examining the growth of *Chlorella pyrenoidosa* during 14 days on water acquired from the natural environment of the upper course of the Vistula River. Because chemical and bacteriological pollutants had already been examined, it was possible to describe regression model estimating relationship between the growth of *Chlorella* and the pollutants [20].

In this study, two criteria were used to evaluate the influence of selected chemical components (present in water sampled each month from the Goczałkowice Reservoir), on the *Chlorella vulgaris* culture applied in a short-term bioassay. The former one monitored changes in the coefficient of the cells division (K_d) in the investigated cultivation system, showing the biomass increase in a single life cycle (24 h). The other one evaluated changes in the absorbance (measured for 10 h, at the wavelength of 680 nm) of the culture liquid media and recalculated per 10^6 cells (showing the growth rate of algae and changes of their metabolic activity).

Table 1

Influence of the Goczałkowice Reservoir's water quality in particular months on the biomass increase during the *Chlorella vulgaris* life cycle

Culture	K_d±SD	K_{dr}±SD	Test t for one test sample (*p)	ANOVA (p)
Control	11.7±1.1	1.00		
June	13.0±0.9	1.09±0.08	0.021	0.1158
July	13.7±1.3	1.02±0.10	0.548	
August	12.4±0.8	1.05±0.07	0.081	
September	10.6±0.6	0.99±0.06	0.596	
October	11.3±0.7	1.06±0.06	0.047	

K_d – arithmetical mean of the coefficient of the cell division, K_{dr} – arithmetical mean of relative coefficient of the cells division, SD – standard deviation, *p – comparison of the arithmetical means of the K_{dr} calculated for the algae cultured in a liquid media supplemented with water received monthly from all sampling points within the Goczałkowice Reservoir with the control culture growing in a liquid medium prepared on distilled water (K_{dr} = 1).

It has been demonstrated (Table 1) that the average value of the cells division coefficient (K_d) calculated for the control liquid culture medium (prepared with distilled water) was 11.7, whereas K_d values obtained for algae cultures growing on water sampled from the Goczałkowice Reservoir varied from 10.6 (September) to 13.7 (July). It must be mentioned that a big flood took place in July 1997, influencing the quality and quantity of pollutants reaching the reservoir waters. The sum of rainfalls in this month attained 278 mm, while the average value calculated for years 1987–1996 was 74.4 mm [21]. Additionally high number of mesophilic bacteria was detected, indicating water pollution with faeces. Besides, the concentration of silica present in the Goczałkowice waters in July 1997 was increased due to stream from rocks in the reservoir basin. On the other hand, the concentrations of nitrates higher than in remaining investigated months could be an effect of fertilizers being washed out from farmlands. At the same time, phenol was not found in the reservoir water, while concentrations of anionic detergents, chlorides and sulfates in the Goczałkowice Reservoir were lower (Fig. 3) [21].

Values of the relative coefficients of the cells division (K_{dr}) were in general higher than one (Table 1). The statistical analysis (ANOVA) indicated no differences between K_{dr} calculated for algae cells grown in the presence of waters sampled from the Goczałkowice Reservoir in particular months (Table 1). To compare the arithmetical means of the K_{dr} values calculated for the algae cultures grown in a liquid media supplemented with waters received each month from all measuring points within the Goczałkowice Reservoir, with the control culture grown in a liquid medium prepared on distilled water (K_{dr} = 1), the test *t* for one test sample has been used. Significant differences have been detected between K_{dr} values calculated for the algae cultures growing in liquid media containing water samples collected from the Goczałkowice Reservoir in June and October 1997 as compared with the control culture of algae growing in liquid medium prepared on distilled water (Table 1).

The rate of algae growth and changes in their metabolic activity have been estimated based on changes in the absorbance at the wavelength of 680 nm during the light phase of the algae growth (first 10 h of the life cycle). Absorbances were calculated as compared with number of 10^6 cells per 1 cm^3 growth. It must be emphasized that the response within 10 h of exposure of investigated algae culture to a toxic environment is 2–4 times quicker as compared with other biotests described in literature, including a rapid algal toxicity assay using chlorophyll fluorescence measurements in *Chlorella kesslari* algae after their 24 h exposure. A two times quicker response seems to be a great advantage over other bioassay methods [22].

Such values are indicators of activities of enzymes participating in the synthesis of chemical compounds, mostly chlorophyll pigments absorbing light at the wavelength used. In asynchronous cultures of algae cells, measuring the absorbance at the wavelength of about 680 nm is used as a convenient indirect method of assessing the biomass increase [23, 24].

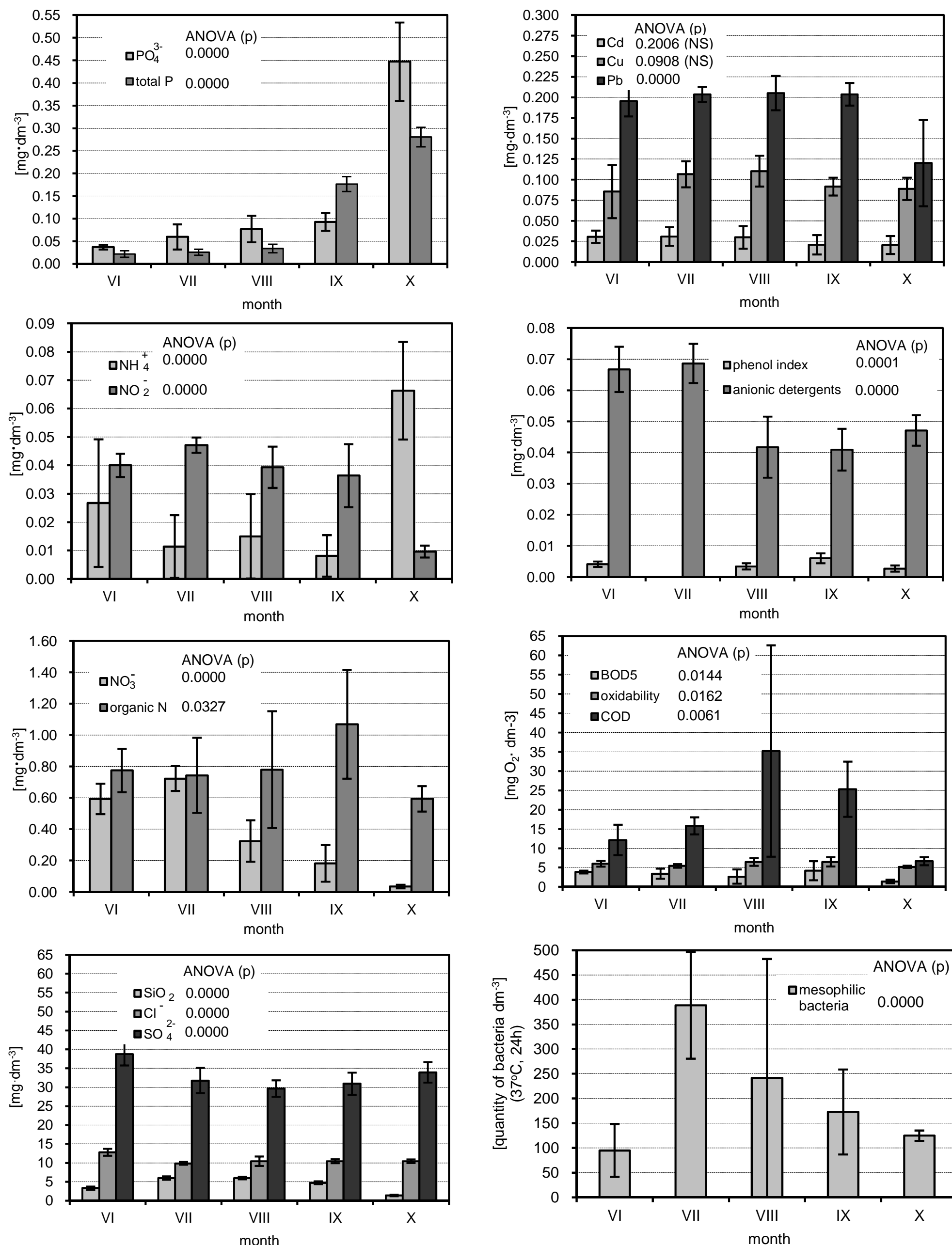

Fig. 3. Selected average levels of water pollution in the Goczałkowice Reservoir and the statistical evaluation (ANOVA) of their diversity from June till October (COD – Chemical Oxygen Demand, BOD5 – Biochemical Oxygen Demand after 5 days)

However, Griffiths et al. [25] claim that this method is fraught with a relatively large error (approximately 52%), resulting from, inter alia, different morphology of cells and their pigment content. In synchronous culture of *Chlorela vulgaris*, cells were brought to the same stage of development through cyclical changes in lighting and strictly controlled conditions for their growth, i.e. a constant temperature, specific illuminance per unit of culture reactor area, stable cell concentration in 1 cm^3 of the culture medium, aeration of culture by purified air enriched in the bright phase of the culture by 2% CO_2, etc. Therefore, the optical density of the cell suspension measured at $\lambda = 680$ nm in the first 10 h of life cycle can be not only a measure of the increase in biomass but most of all in biological activity of a single cell. This cell begins its life cycle when the light is switched on and do not divide during the whole light phase but passes through the successive stages of development – from aplanospor to the mother cell, resulting in a constant number of cells in 1 cm^3 of culture medium.

Table 2

Statistical analysis of linear regression (ANCOVA, $p = 0.0058$) for dynamics of the monthly changes in mean values of recalculated absorbance for *Chlorella vulgaris* cultures growing in a liquid media with water from the Goczałkowice Reservoir

Month	Equation of the linear regression	Correlation coefficient
June	$y = 0.0341x + 0.2058$	0.9967
July	$y = 0.0371x + 0.1753$	0.9933
August	$y = 0.0361x + 0.1821$	0.9963
September	$y = 0.0348x + 0.2110$	0.9960
October	$y = 0.0313x + 0.2193$	0.9958

For the evaluation of the influence of Goczałkowice water pollution on the above parameters calculated for each month, the analysis of covariance (ANCOVA) has been used. As the zero-hypothesis (H_0), the parallelism has been accepted between straight lines representing the dynamics of the algae growth and their bioactivity. It has been shown that obtained straight lines were not parallel ($p = 0.0058$, Fig. 4, Table 2). These results point to differences between the waters compositions in the Goczałkowice Reservoir in the months studied. Results of the homogeneity test of the regression coefficients showed that the June, July and August water samples influenced the algae growth and their metabolic activity to a similar extent ($p > 0.167$) whereas the October water samples showed different effects ($p < 0.05$). It may be caused by changes in the waters quality in the Goczałkowice Reservoir resulting from the temperature decrease typical of lakes and simultaneous decrease of the metabolic activity of *in situ* living algae. Results of the homogeneity test of the regression coefficients point at the signif-

icant differences ($p = 0.0088$) in the dynamics of the increase of metabolic activity in *Chlorella vulgaris* cells used as a bioindicator of waters quality in June and July.

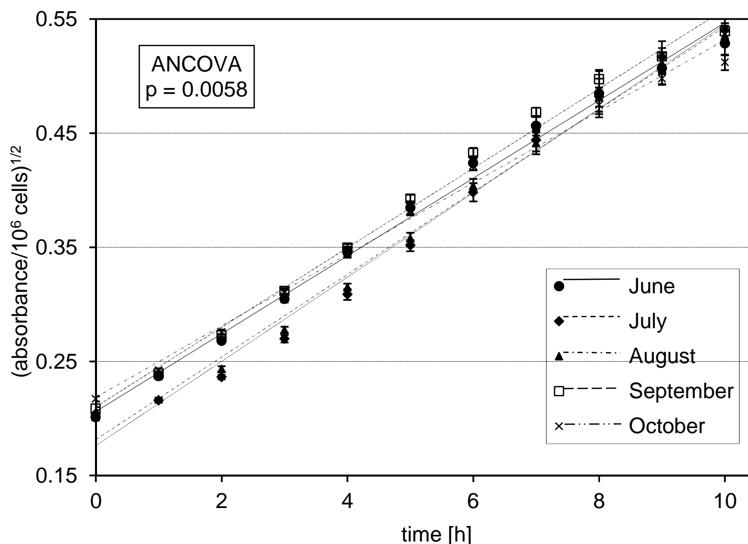

Fig. 4. Linear regression of the dynamics of growth of *Chlorella vulgaris* cells cultured in a liquid media with water from the Goczałkowice Reservoir; changes of metabolic activity are shown as recalculated values of (absorbance/10^6 cells)$^{1/2}$

Changes in the metabolic activity of cells of *Chlorella vulgaris* algae grown in the synchronous culture allow one to distinguish two specific periods within the study period, i.e. from June to October 1997. The former one is related to specific flood conditions in July, and the latter one to autumnal water quality changes due to pollution by discharge from the greatest fish pond complex in the Central Europe (over 9 km^2 of the surface area).

4. CONCLUSIONS

The dynamics of changes in the absorbance values measured in *Chlorella vulgaris* cells cultured in a liquid medium during first 10 h of the algae life cycle as well as calculated values of the coefficients of the algae cells division in the synchronous culture may be useful as the sensitive, inexpensive and quick method of monitoring changes in waters quality in lakes.

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