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OPTIMIZATION OF MEASUREMENT ENZYME ACTIVITY USING FLUOROGENIC SUBSTRATES IN WATER

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Abstract

Investigations have been carried out aiming at reaching the best method for enzymatic activity measurements, with use of fluorogenic model substrates. Absorption and emission spectra have been determined. It has been found out that the fluorescence intensity does not depend on the length of the excitation wave. The findings cover the influence of temperature, pH, and $HgCl_2$ have on the fluorescence.

Key words: fluorogenic substrates, enzymatic activity, temperature, pH, chloride mercury, brackish water

INTRODUCTION

Organic matter which occurs in water basins exist in two forms: dissolved organic matter (DOM) and particulate organic matter (POM). In water ecosystems, DOM which makes up about 90% of total organic matter, is the main source of carbon and energy for heterotrophic bacteria (Műnster and Chróst 1990). Biopolimers make significant percentage of DOM, which being macromolecules, are too big particles to be incorporated directly by bacterial cells inside them (Chróst and Velimirov 1991; Hoppe 1993). Hence, the bacteria have the ability to synthesise a wide range of extracellular enzymes which are able to depolimerise various high molecular weight polymeric compounds (Mudryk and Donderski 1992; Mudryk 1998). Hydrolytic extracellular enzymes known also as exoenzymes present in the periplasm and cell envelope of bacteria are of fundamental importance for organic matter decomposition rate in water ecosystems, and its flow through the microbiological cycle (Meyer-Reil 1981; Martinez *et al.,* 1996).

However, methodology problems relating to the accurate measurement of microorganisms enzymatic activity level in water basins have not yet been fully solved

(Marxsen and Witzel 1993). Investigations that have currently been underway focus on two aspects: a quality one aiming at defining the accurate percentage of particular enzyme type synthethising microorganisms, and a quantity one - involving determining the amount of substrate decomposed by enzymes in particular water volume and within a particular period of time (Kjelleberg and Hakansson 1977; Kim and Hoppe 1986).

There are different methods for defining the quantity of enzyme activity level in water basins. One of the most accurate is the one that involves fluorogenic tracer substrates (Hoppe 1983; Marxsen and Witzel 1993; Martinez *et al.,* 1996) These substrates contain an artificial fluorescent molecule and one or more natural molecules, linked by a specific binding and fluorescence is observed after enzymatic splitting of the complex molecule (Kim and Hoppe 1986; Hoppe 1993). There are different variations of that method that can be found in the literature which differ between one another by fluorescence molecules that have been used. In microbiological enzymatic studies coumarin and its derivatives have been widely used (Kim and Hoppe 1986; Marxsen and Witzel 1990; Hoppe 1993; Vrba *et al.,* 1993; Christian and Karl 1995 a,1995 b; Martinez *et al.,* 1996). Coumarins are a group of compounds occurring in plant tissues and are particularly interesting due to their powerful luminising and lasering qualities (Koch 1985). Umbelliferone (7- hydroxycoumarin) is one of coumarin related substances (Heldt *et al.,* 1995). This compound shows two fluorescence sequences in mean polar solvents, which tends to be interpreted as related to a common induced form and to phototautomer (Grzywacz *et al.,* 1978).

Coumarins are different only in terms of substituents but possess similar outlines of absorption and fluorescence spectra which undergo changes when affected by the environment pH oscillations. Higher pH values provide a batochrome effect and spectrum intensity shifts (Grzywacz and Taszner 1977). Batochrome shifts to coumarin absorption and fluorescence spectra and their derivatives may also depend on the solvent polarity (Czajko and Kozma 1981). The present paper presents results of examinations carried out with the use of two heavily fluorescent derivatives of coumarin: 4-methyl - umbelliferone (MUF) and 4-methyl - coumarin-7-amide (MCA), which, when combined by suitable organic substrates, provide only slight fluorescence (Marxsen and Fiebig 1993).

The objective of the paper was to reach optimum in the research method of precise determining of enzymes activity level in water of estuarine Lake Gardno. Particularly important was to define the influence of the following physical and chemical factors on fluorescence intensity: the excitation wave, pH, temperature and $HgCl_2$.

MATERIAL AND METHODS

The investigations were carried out with the use of pure fluorescent compounds of 4-methyl - umbelliferone (MUF) and 4-methyl - coumarin-7-amide (MCA) and also two organic substrates with fluorescent tracer of MUF-butyrate and MUF-N-acetyl-β-D-glucosaminide (Sigma). The compounds were dissolved at 20°C in methylcellosolve (ethylene glycol monomethyl ether Sigma) prior to the experiment to eventually reach a concentration of 10 mM dm^{-3}. Thus prepared solutions of fluorescence

compounds were kept in the dark at -25°C (Kim and Hoppe 1986; Scholz and Marxsen 1996). Working solutions were made before the experiment by the dilution of stock solutions with of tris/HCl buffers, which create the optimal pH for the desired enzyme reaction (Kim and Hoppe 1986; Hoppe 1993).

Absorbance was determined in pure solutions of 100 μM dm^{-3} MUF and MCA. The influence of inducing light wavelength on fluorescence level was defined at the same time.

Examinations of temperature influence on fluorescence level were done with the use of 100 μM dm^{-3} MUF and MCA solutions, at 365 nm excitation wavelength. Peltier head was used for temperature stabilising.

MUF-butyrate and MUF-N-acetyl-β-D-glucosaminide solutions 100 μM dm^{-3} exposed to high temperature for fluoresceine molecules release, were used for defining the effect of pH on fluorescence level. In the course of the investigations a series of tris/HCl buffers were used, within pH range of 4.8 to 9.0. Fluorescence was measured at an excitation wavelength setting of 365 nm and an emission setting of 445 nm.

MUF-butyrate of 100 μM dm^{-3} was used for determining the pH influence on self -dissolution of fluorescence substrates. Three solutions of MUF-butyrate with pH 5.0, pH 7.4 and pH 8.2 were prepared. The examinations were going on for 90 minutes. Fluorescence was measured at 365 nm (excitation) and 445 nm (emission).

Investigations concerning $HgCl_2$ influence on fluorescence intensity were carried out on MUF and MCA solutions of 100 μM dm^{-3} and pH 7.4. Saturated $HgCl_2$ from 1 to 6 μl per 1 cm^3 was added to 3 cm^3 of MUF and MCA. The control samples were prepared with no mercury ions added. Fluorescence was induced by 365 nm excitation wavelength.

The absorption spectra were measured on a Specord M. 40 (Carl Zeiss Jena) spectrophotometer. Fluorescence spectra were measured upon frontal excitation and observation of the sample fluorescence using the spectrofluorometer described by Kawski *et al.,* (1994) and the results were corrected for the spectral sensitivity of the equipment. The excitation source was a 1000 W xenon lamp. The wave lengths required for excitation and emission were selected using prismatic monochromators (Carl Zeiss Jena). A Hamamatsu R 928 photomultiplier was used as a detector.

RESULTS

Fig. 1 presents the research results concerning the relation between fluorescence and the excitation wavelength. The data provide information on no dependence of spectrum shape of the used fluorescence molecules on the exciting wavelength. In the case of MUF the maximum absorbance was recorded at 318 nm wavelength and the maximum fluorescence intensity at 445 nm wavelength. For MCA the highest absorbance level was recorded at 345 nm wavelength and a maximum fluorescence intensity at 425 nm wavelength.

Data on temperature influence on MUF and MCA fluorescence intensity is presented in Fig. 2. In the case of MUF a heavy fluorescence fading was recorded along with temperature increase, whereas in the case of MCA the fluorescence intensity was not affected by that temperature.

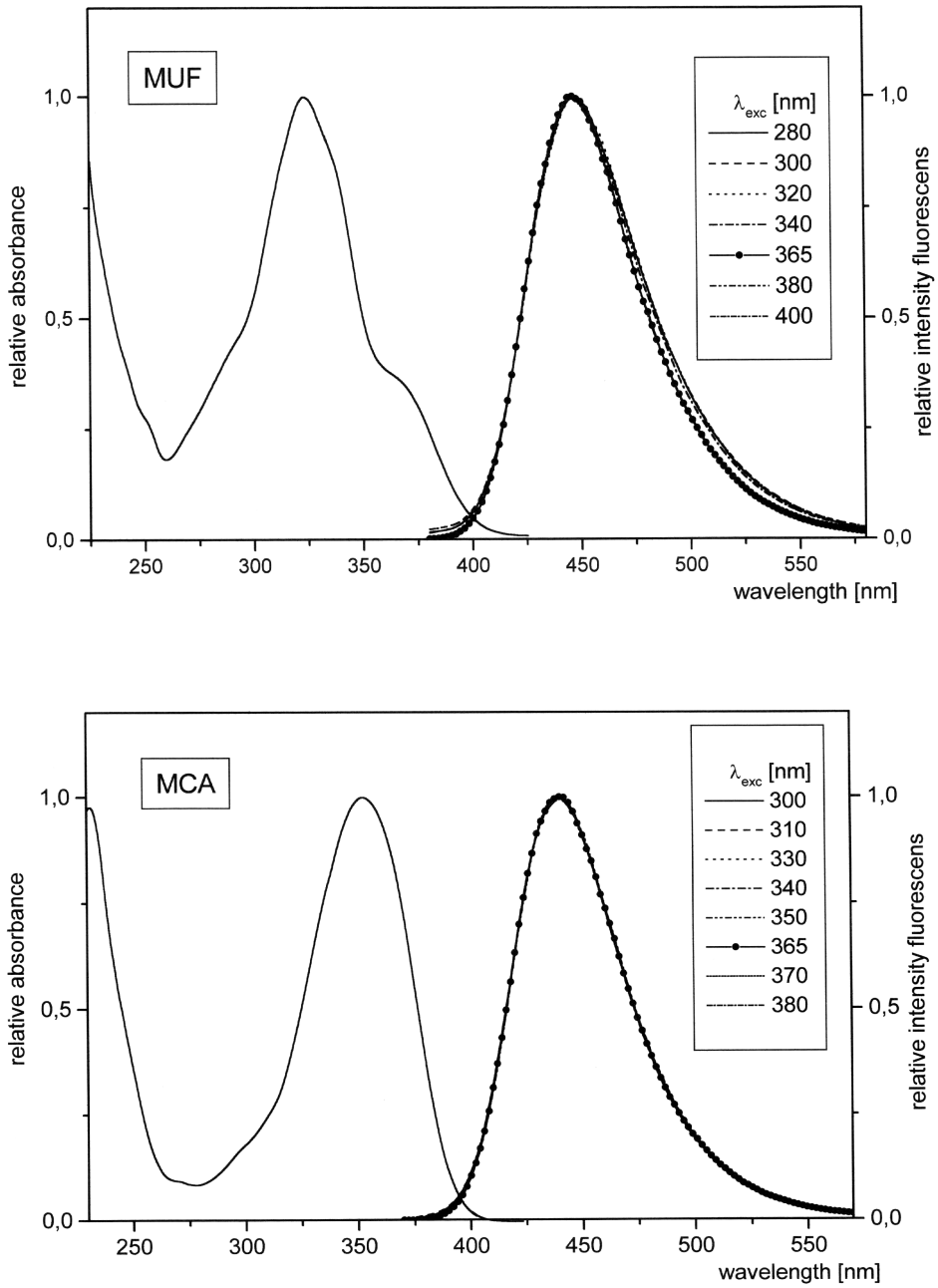

Fig. 1. Relation between relative fluorescence intensity and the excitation wavelength

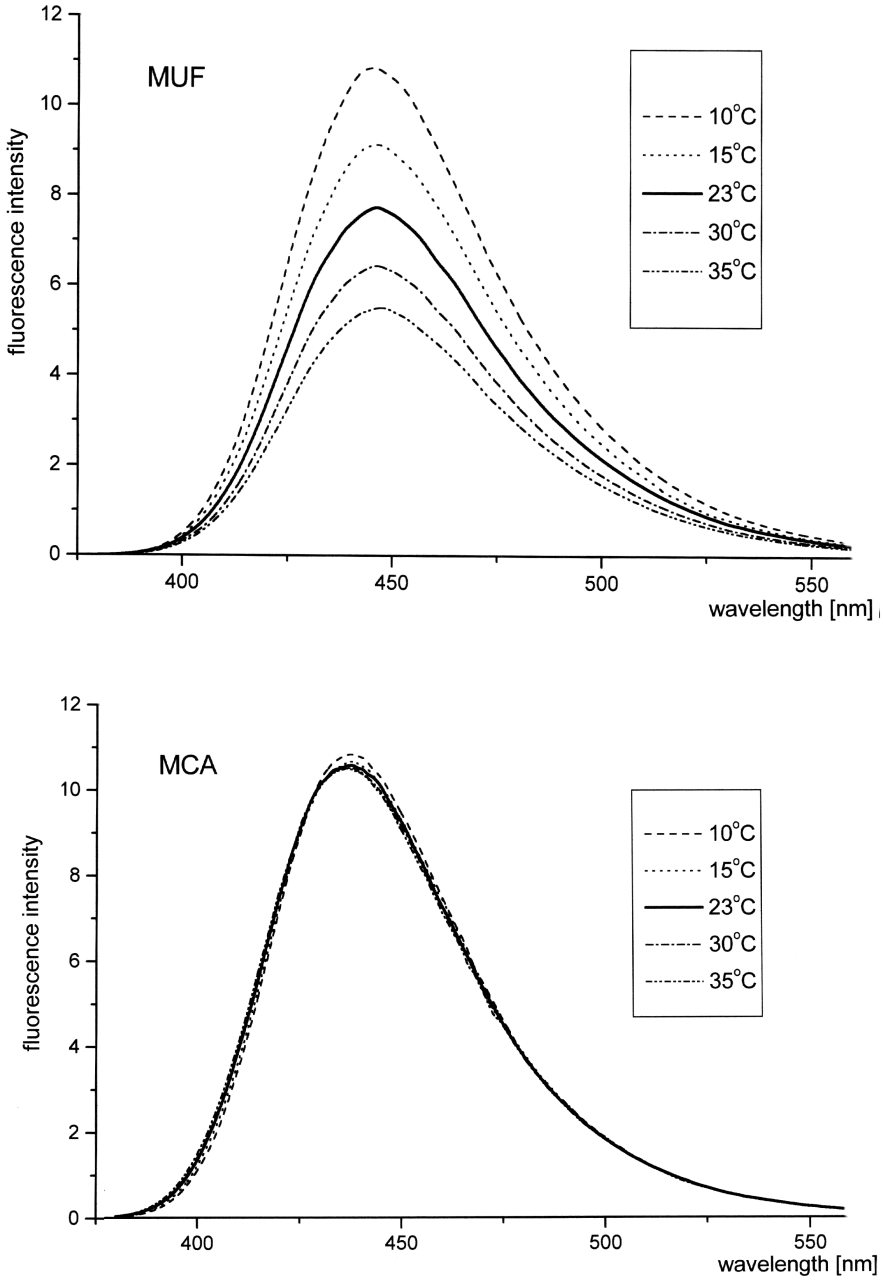

Fig. 2. Influence of temperature on MUF and MCA fluorescence intensity

Fig. 3 presents data concerning the relation between fluorescence intensity and pH of the solution containing fluorescence tracer substrate. A slight fluorescence intensity increase was recorded in 4.8-8.2 pH range. Above pH 8.2, the fluorescence of MUF-butyrate and MUF-N-acetyl-β-D-glucosaminide rose rapidly.

Data on the dependence of fluorescence substrate self - dissolution MUF-butyrate on the environment pH present Fig. 4. As it was proved, the pH increase triggered a more intensive process of self - dissolution which eventually released fluoresceine particles.

Fig. 5 provides information on the influence of mercury chloride on the fluorescence level. The findings proved that an increase in mercury ions concentration was accompanied by a heavy fading of MUF fluorescence. On the other hand, no dependence of various $HgCl_2$ concentrations on MCA fluorescence level was found.

DISCUSSION

Recently many investigators have applied fluorogenic substrates for direct *in situ* measurement of bacterial extracellular enzymatic activities in water ecosystems (Hoppe 1983; Kim and Hoppe 1986; Vrba *et al.,* 1993; Martinez *et al.,* 1996; Scholz and Marxsen 1996). The majority of studies on enzymatic activity measured by means of fluorogenic tracer substrates provided information that many physical and chemical factors play an important role in those measurements (Boon 1989; Chróst and Velimirov 1991; Christian and Karl 1995a, 1995 b; Mudryk 1998).

The data obtained from our investigations pointed to the fact that there was no influence of excitation wave length on the MUF and MCA solutions fluorescence intensity. This facilitated excitation by any wavelength which came within absorbance range. According to the methodology recommended by Kim and Hoppe (1986) a 365 nm wavelength was applied in the present study. However, Marxsen and Fiebig (1993) maintain that it is more favorable to use 380-390 nm wavelengths for MUF and MCA fluorescence excitation. It prevents from an error caused by residual fluorescence of non-decomposed substrates, which normally gets shorter with excitation wave length increase. However, there is the threat of a change that may occur to the fluorescence spectrum shape.

According to the present data, temperature may significantly affect the MUF fluorescence level. Hence, is a need to keep the temperature constant in a very precise way while the measurements are being done. The available literature often emphasises the necessity of maintaining temperature on a constant level in the course of sample incubation (Boon 1989; Christian and Karl 1995b; Marxsen and Fiebig 1993). At the same time, Martinez *et al.,* (1996) points to the necessity of stabilizing the temperature also at the time of measurements.

Many investigations point to the fact that it is of major importance to accurately buffer the samples under analyses as pH has a fundamental influence on the fluorescence level (Grzywacz and Taszner 1982; Boon 1989; Marxsen and Witzel 1990; Hoppe *et al.,* 1993; Vrba *et al.,* 1993; Scholz and Marxsen 1996). Our results also confirmed the pH increase causing a multiplied fluorescence intensity. For this reason, the amount of fluoresceine released from substrates is usually measured at pH range from 10.0 to 10.5 (Hoppe 1983, 1986; Marxsen and Fiebig 1993).

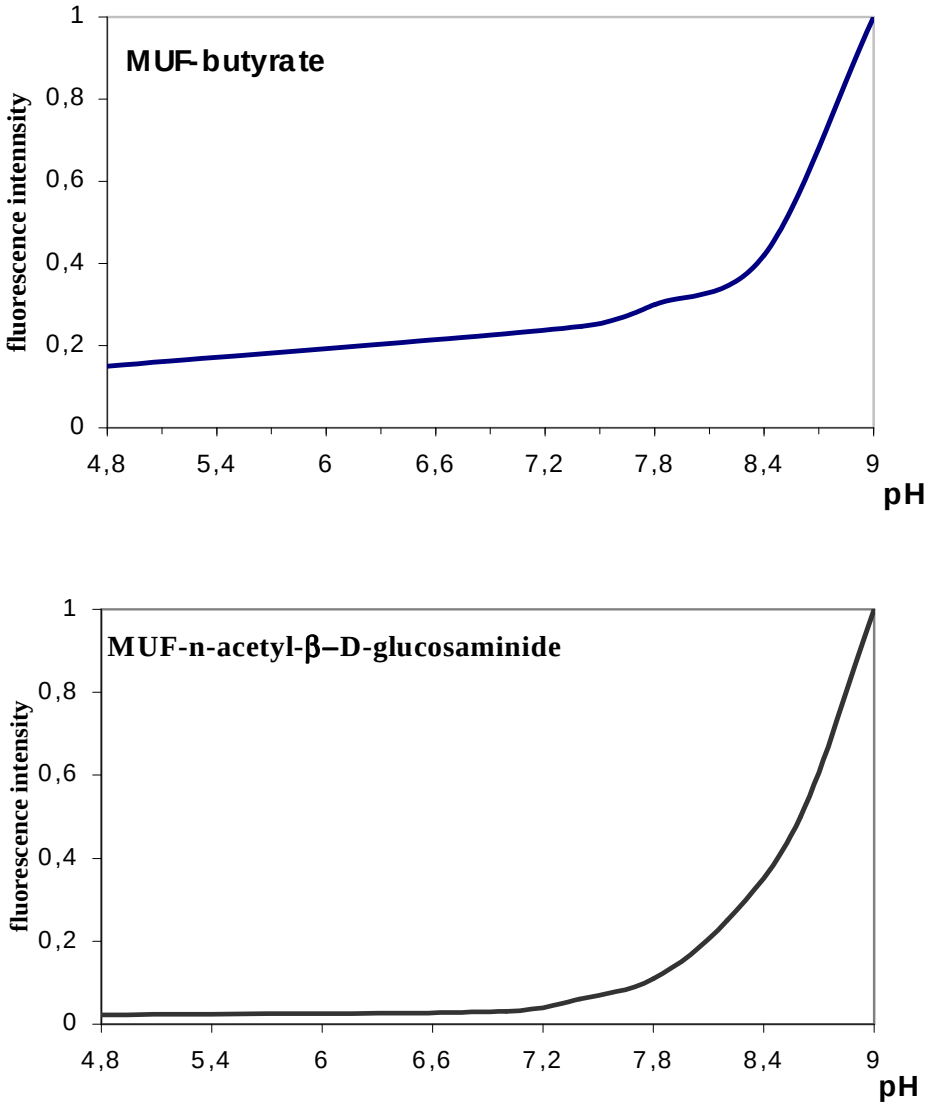

Fig. 3. Effect of pH on fluorescence level of fluorogenic organic substrates

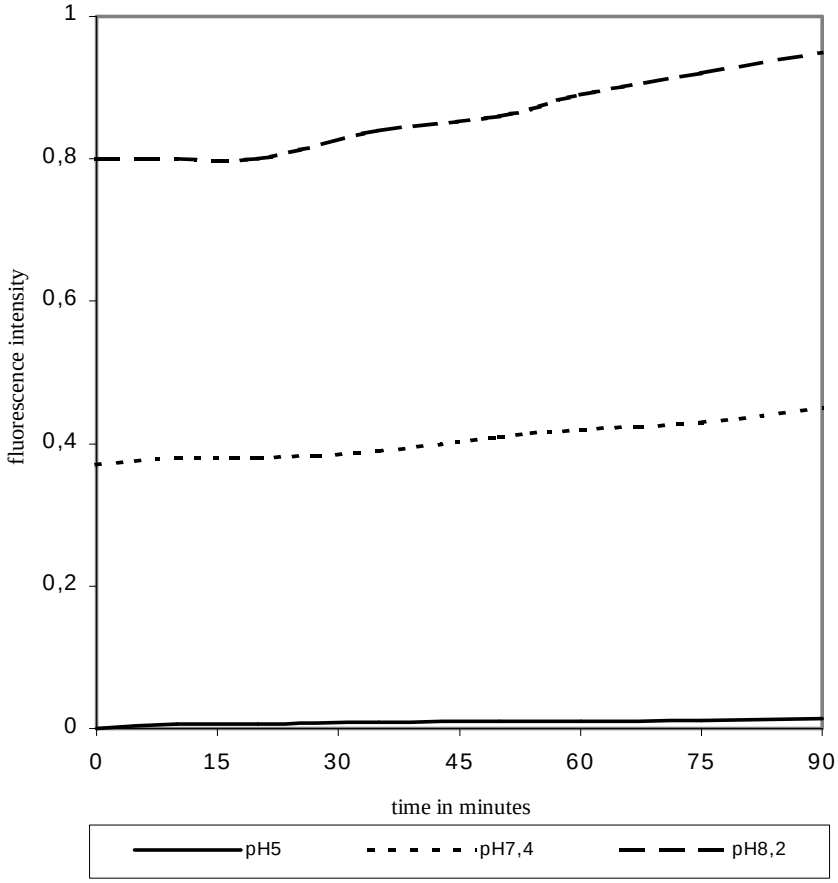

Fig. 4. Influence of the ionic strength on self-dissolution of fluorescence substrates

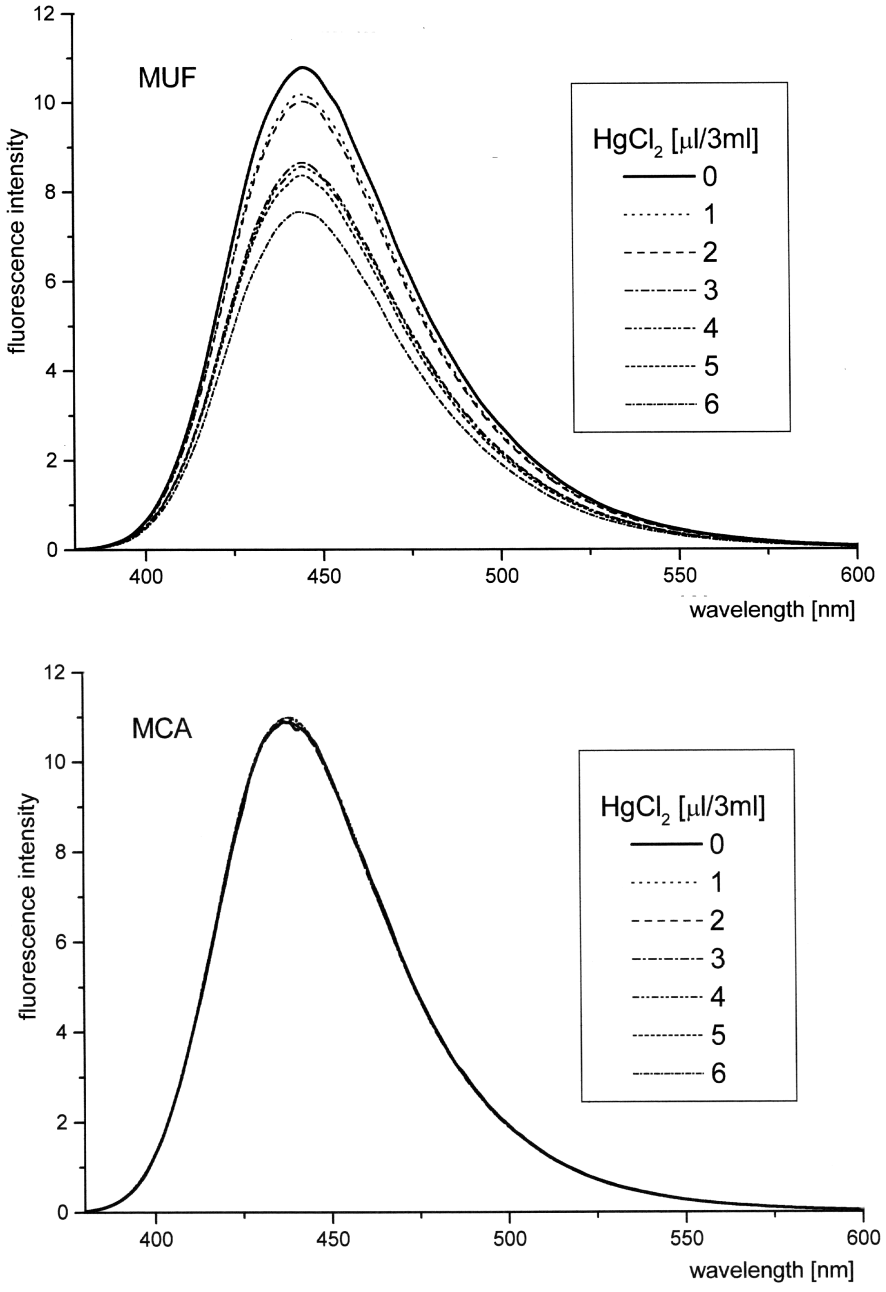

Fig. 5. Effect of different concentrations mercury chloride on fluorescence level

However, Christian and Karl (1995 b) warn against the danger of enzyme inactivation at such high pH. Besides, in heavily alkaline solutions even small pH oscillations occurring between particular samples may significantly affect the fluorescence level. The authors of the present study found out that also the self-dissolution of fluorogenic tracer substrates may increase along with the pH increase in a solution. Such phenomenon may significantly twist the reliability of the data that had been arrived at as a result of time-consuming incubations going on for many hours, especially when done in solutions with high pH (Marxsen and Witzel 1993).

Examinations of enzymatic activity in water, which are carried out outside laboratory, must be followed by sample preservation once the incubations have been completed. The optimum reached within the preservation method should ensure the result constancy over the longest possible period of time (Christian and Karl 1995a). Literature provides examples of commonly used sample preservation methods such as stopping enzymatic reactions by sample boiling through for several minutes (Marxsen and Witzel 1990; Hoppe 1993) or adding saturated $HgCl_2$ solution (Christian and Karl 1995a). The data presented in the present paper prove that mercury chloride may be used as a preservative with no limitations in the case of MCA stained substrates. But, on the other hand, $HgCl_2$ applied to MUF stained substrates may result in gradual fluorescence fading, which obviously imposes restrictions as to its usage as a preservative.

The authors of the present paper maintain that their studies enabled reaching an the optimalization in further measurements of enzyme activity in water samples of estuary Lake Gardno.

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OPTYMALIZACJA POMIARU ENZYMATYCZNEJ AKTYWNOŚCI W WODZIE Z UŻYCIEM SUBSTRATÓW FLUOROGENNYCH

Streszczenie

Celem przeprowadzonych badań było zoptymalizowanie parametrów pomiarów aktywności enzymatycznej w naturalnych próbach wody przy użyciu modelowych substratów fluorogennych. W trakcie wykonywanych pomiarów oznaczano poziom absorpcji i emisji. Stwierdzono, że intensywność fluorescencji jest niezależna od długości fali wzbudzania. Natomiast istotny wpływ na poziom fluorescencji miała temperatura, pH oraz stężenie $HgCl_2$.