UDC 577.11:576.311.31:57.086]:606:628.3 https://doi.org/10.15407/biotech10.06.028

WASTEWATER COMPONENTS EFFECT ON METACHROMASIA REACTION OF VOLUTIN GRANULES *in vitro*

M. S. Kharchuk
E. N. Gromozova

Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv

E-mail: sithmcx@ukr.net

Received 28.09.2017

Microorganisms that contain the polyphosphates volutin granules take active part in phosphorus and heavy metals removal from the wastewater. The metachromatic reaction is a simple cytochemical method for the detection of these granules. The objective of current research was to study the metachromatic reaction of inorganic polyphosphate with Methylene Blue dye in combination with other components of wastewater (proteins, carbohydrates, metal ions) *in vitro*. It was demonstrated that manifestation of metachromatic coloration depends on the polyphosphate concentration and to a lesser extent, on its chain length. Glucose did not influence metachromasy reaction. At the same time, calcium ions and bovine serum albumin, depending on their concentration, stimulated or inhibited the metachromatic color of the test solutions. Bovine serum albumin, in contrast to calcium ions, had a lesser effect on metachromasy. Thus, the abundant accumulation of polyphosphates and metal cations (as we demonstrated with of Ca^{2+} ions), in microorganisms of activated sludge not always accompanied by a pronounced reaction of metachromasy of the volutin granules. In this regard, the use of other cytochemical methods for the identification of polyphosphate granules is recommended, for example, staining with fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI).

Key words: volutin granules, polyphosphate-accumulating organisms, metachromasia reaction, wastewater treatment.

Currently, the technology of Enhanced Biological Phosphorus Removal (EBPR) is an effective and economically viable method for wastewater treatment from excess phosphorus and cations of heavy metals. EBPR technology is based on the activity of microbial communities of active sludge, which accumulate phosphorus and transform it into volutin granules [1, 2]. These granules include polyphosphates (linear condensed phosphates, linked by P-O-P macroergic bonds), which are able to bind both metal cations and proteins [2].

The earliest and simplest method for identifying of volutin granules is their cytochemical determination using cationic dyes [1–3], such as Methylene Blue. When staining, granules can exhibit the metachromatic reaction. This reaction is a change in the original color of the dye, which is due to the shift of its absorption spectrum toward shorter wavelengths [3–6]. It is believed that the mechanism of metachromasy occurrence involves aggregation (polymerization) of the dye on the polyanion chain, which serves as matrix [5, 6]. The metachromatic reaction of volutin granules can have a different degree of manifestation. Perhaps this is due to polyphosphate metabolism, which depends on the changes in environmental conditions, as has been demonstrated by the example of Chizhevsky-Velhover bio-astronomical effect in the yeast *Saccharomyces cerevisiae* [7].

In connection with this, the aim of this work was to research the metachromatic reaction of Methylene Blue with polyphosphates, in combination with different natural compounds, as possible model components of sewage that affect the composition of the volutin granules.

Materials and Methods

We used aqueous solutions of inorganic polyphosphates with a chain length of 12–18 and 200 phosphate residues (Sigma, USA, Reanal, Hungary) in the final concentrations of 0.05; 0.25; 0.5 and 5 mg/ml. The pH of

solutions was adjusted to neutral by titration with 0.1 N NaOH. The Methylene Blue concentration in samples was 0.05 mg/ml. Glucose, bovine serum albumin and $CaCl_2$ at final concentrations of 0.1 and 1 mg/ml have been used as model components for carbohydrates, proteins and metal cations.

The absorption spectra of Methylene Blue in samples were recorded against water on the DeNovix DS-11 FX+ spectrophotometer (DeNovix Inc., USA).

The effect of medium pH on the spectral shift of Methylene Blue absorption was recorded visually (by changing the color of the solutions) and by spectroscopy approach. To achieve this, test solutions were acidified with 0.1 N HCl to a pH of 2.7 and below, and also alkalinized with 0.1 N NaOH to a pH of 10.8 and higher.

Results and Discussion

It is known that Methylene Blue absorption peaks in dilute aqueous solution are located at 664 nm (α-band) and at 610 nm (β-band), which is characteristic for the dye in monomeric state [6]. The appearance of a small metachromatic band simultaneously with a decrease in the α-band's intensity is characteristic for the dimer or weakly aggregated Methylene Blue, which is manifested by weak metachromasia. Pronounced metachromatic coloring is characterized by disappearance of the α-band and the appearance of intense band at a wavelength of about 550 nm. Such effect is attributed to a highly aggregated dye [6]. In our studies, the absorption spectra of Methylene Blue aqueous solution (0.05 mg/ml) were characterized by a pronounced α-band at a wavelength of about 664 nm and a β-band at 614 nm (Fig. 1–2: *(1, 1′)*), which is in agreement with literature data. The addition of polyphosphate with a chain length of 12–18 phosphate residues at a final concentrations from 0.05 mg/ml to 0.5 mg/ml into a solution of Methylene Blue led to the appearance of a metachromasia reaction (Fig. 1: *(3, 3′)–(5, 5′)*). Further increase in the concentration of polyphosphate results restoration of α- and β-bands and disappearance of the metachromatic band (Fig. 1: *(6, 6′)*). A more intense metachromatic color was observed in the present of polyphosphate with a chain length of 200 phosphate residues (Fig. 2: *(3, 3′) — (5, 5′)*). Absorption spectra from polyphosphates were not recorded (Fig. 1–2: *(2, 2′)*). Thus, the metachromatic reaction depends primarily on the concentration of the polymer, and then on the degree of its

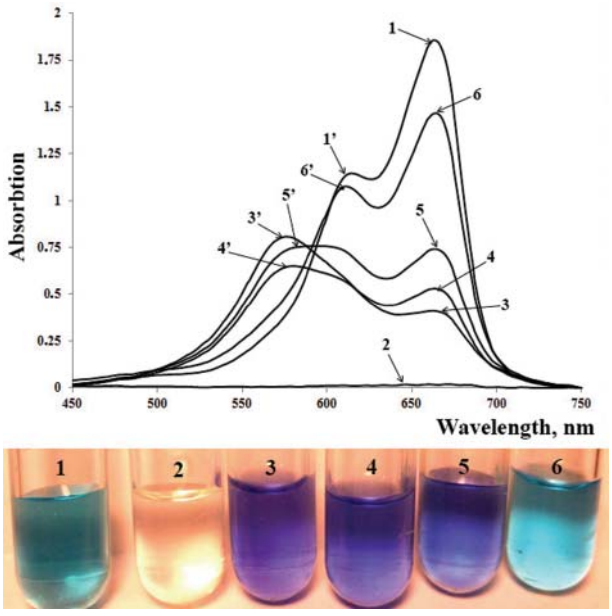

Fig. 1. **Absorption spectra (λ, nm) of Methylene Blue (MB, 0.05 mg/ml) and its complexes with polyphosphate with a chain length of 12–18 phosphate residues ($PolyP_{12-18}$) of different concentrations:**

1) MB (*1* — 664 nm, *1′* — 614 nm);
2) 2 mg/ml of $PolyP_{12-18}$ (2 — there is no absorption maximum);
3) MB + 0.05 mg/ml of $PolyP_{12-18}$ (*3* — 664 nm, *3′* — 575 nm);
4) MB + 0.25 mg/ml of $PolyP_{12-18}$ (*4* — 665 nm, *4′* — 580 nm);
5) MB + 0.5 mg/ml of $PolyP_{12-18}$ (*5* — 665 nm, *5′* — 590 nm);
6) MB + 5 mg/ml of $PolyP_{12-18}$ (*6* — 665 nm, *6′* — 610 nm)

polymerization, which agrees with the earlier published findings [3, 5, 8, 9]. It is known that polyphosphates with a chain length of less than 10 phosphate residues have no metachromasy [3, 5, 9]. This is due to the number of anionic groups on the polymer, which play an important role in the binding of Methylene Blue and its aggregation [6, 9]. On the other hand, the metachromatic reaction may depend on the polymer state (sol-gel transitions) and conformation (at a distance between reaction groups equal to/or less than 5 Å) [4]. An increase in the concentration of the polyanion leads to the redistribution of aggregated Methylene Blue on anionic groups of polymer molecules [6]. This in turn, this results in restoration of α- and β-bands and to the disappearance of metachromatic band [6, 8].

It is known that solution pH change can influence the aggregation of Methylene Blue and, a result in appearance of metachromasy [3, 4]. It has been shown that acidification and alkalization of dye solutions (0.05 mg/ml) did not lead to a metachromatic shift of its absorption spectrum (Fig. 3: (*1–1′; 3–3′*)). The addition of polyphosphate at a final concentration of 0.05 mg/ml promoted the appearance of a metachromatic reaction at neutral pH (Fig. 3: (*6–6′*)). The metachromatic color of methylene blue solution with polyphosphate was preserved both at acidic (Fig. 3: (*4–4′*)) and alkaline (Fig. 3: (*7–7′*)) media. However, at pH below 2.7 (Fig. 3: (*5–5′*)) and above 10.8 (Fig. 3: (*8–8′*)), it disappeared. Thus, a pH change of about 2.7 to 10.8 has practically no effect on the metachromatic reaction.

The addition of glucose into the reaction mixture of Methylene Blue with polyphosphate did not lead to a shift in the absorption spectra (Fig. 4). Thus, glucose does not affect the color of the solutions.

Unlike glucose, calcium chloride exerted influence on the metachromatic reaction in solutions of Methylene Blue with polyphosphate (Fig. 5). Metachromatic coloring could either appear or disappear depending on the ratio of $CaCl_2$ and polyphosphate concentrations in solution. $CaCl_2$ contributed to the appearance of metachromatic reaction if its ratio to polyphosphate was 1: 5 (Fig. 5: (*5–5′*)), respectively. On the contrary, an increase in calcium chloride concentration of was associated with the, metachromasy inhibition (Fig. 5: (*4–4′; 6–6′; 7–7′*)). It is known that polyphosphates have a well-expressed ability to

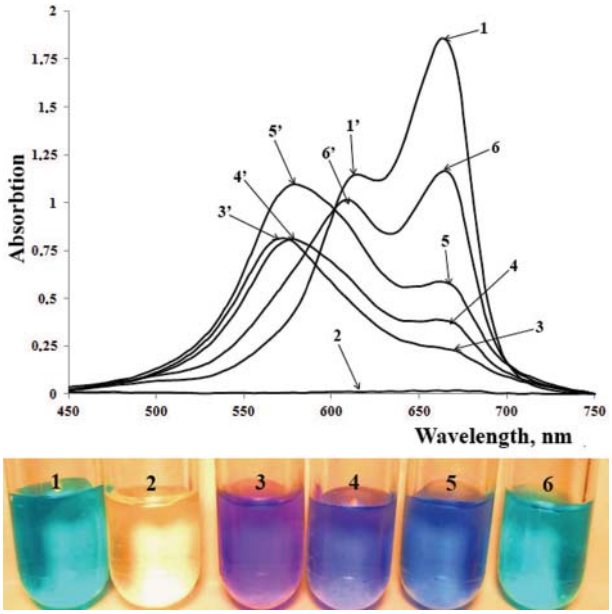

Fig. 2. **Absorption spectra (λ, nm) of Methylene Blue (MB, 0.05 mg/ml) and its complexes with polyphosphate with a chain length of 200 phosphate residues ($PolyP_{200}$) of different concentrations:**
1) MB (*1* — 664 nm, *1′* — 614 nm);
2) 2 mg/ml of $PolyP_{200}$ (2 — there is no absorption maximum);
3) MB + 0.05 mg/ml of $PolyP_{200}$ (*3* — 664 nm, *3′* — 570 nm);
4) MB + 0.25 mg/ml of $PolyP_{200}$ (*4* — 664 nm, *4′* — 675 nm);
5) MB + 0.5 mg/ml of $PolyP_{200}$ (*5* — 664 nm, *5′* — 580 nm);
6) MB + 5 mg/ml $PolyP_{200}$ (*6* — 664 nm, *6′* — 610 nm)

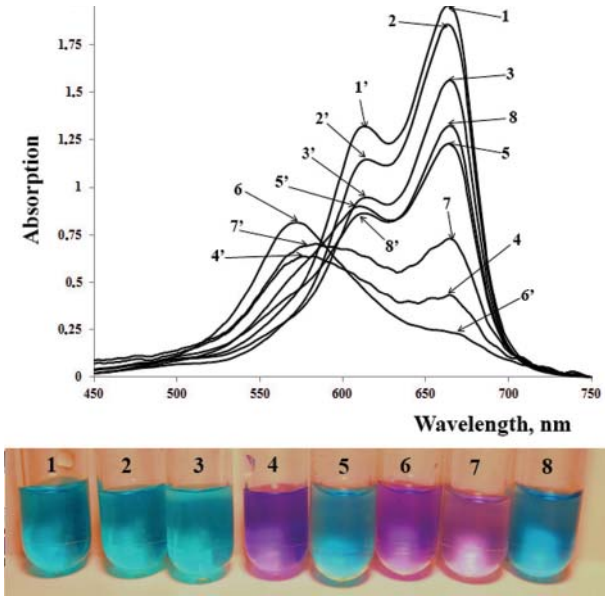

Fig. 3. **Absorption spectra (λ, nm) of Methylene Blue (MB, 0.05 mg/ml) and its complexes with polyphosphate ($PolyP_{200}$, 0.05 mg/ml) at different pH:**
1) pH 2.7 MB (*1* — 664 nm, *1′* — 614 nm);
2) pH 7.1 MB (*2* — 664 nm, *2′* — 614 nm);
3) pH 10.8 MB (*3* — 664 nm, *3′* — 614 nm);
4) pH 2.7 MB + $PolyP_{200}$ (*4* — 664 nm, *4′* — 575 nm);
5) <pH 2.7 MB + $PolyP_{200}$ (*5* — 664 nm, *5′* — 610 nm);
6) pH 7.1 MB + $PolyP_{200}$ (*6* — 664 nm, *6′* — 570 nm);
7) pH 10.8 MB + $PolyP_{200}$ (*7* — 664 nm, *7′* — 580 nm);
8) > pH 10.8 MB + $PolyP_{200}$ (*8* — 664 nm, *8′* — 610 nm)

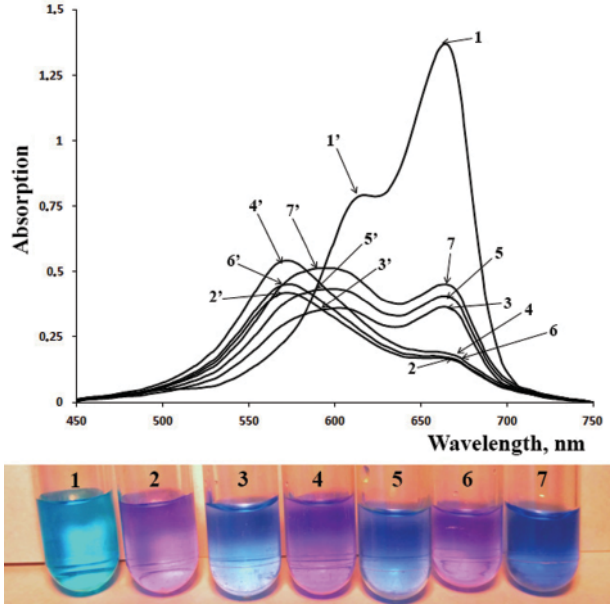

Fig. 4. **Absorption spectra (λ, nm) of Methylene Blue (MB, 0.05 mg/ml) and its complexes with polyphosphate with a chain length of 200 phosphoric residues ($PolyP_{200}$) and glucose (Gl) of different concentrations:**
1) MB (*1* — 664 nm, *1′* — 614 nm);
2) MB + 0.05 mg/ml of $PolyP_{200}$ (*2* — 664 nm, *2′* — 570 nm);
3) MB + 0.5 mg/ml of $PolyP_{200}$ (*3* — 664 nm, *3′* — 600 nm);
4) MB + 0.05 mg/ml of $PolyP_{200}$ + 0.1 mg/ml of Gl (*4* — 664 nm, *4′* — 570 nm);
5) MB + 0.5 mg/ml of $PolyP_{200}$ + 0.1 mg/ml of Gl (*5* — 664 nm, *5′* — 600 nm);
6) MB + 0.05 mg/ml of $PolyP_{200}$ + 1 mg/ml of Gl (*6* — 664 nm, *6′* — 570 nm);
7) MB + 0.5 mg/ml of $PolyP_{200}$ + 1 mg/ml of Gl (*7* — 664 nm, *7′* — 600 nm)

form complexes with various substances, often with the participation of divalent metals, in particular with Ca^{2+} and Mg^{2+} [2]. Thus, calcium ions influence on the manifestation of the methachromatic color of solutions via the direct competition with the dye for binding sites.

Unlike calcium chloride, bovine serum albumin had less pronounced effect on the metachromatic reaction (Fig. 6). Only high concentration of protein added led to disappearance of the metachromatic band of the absorption spectrum of Methylene Blue (Fig. 6: (*6*–*6′*)). It is known [10] that bovine serum albumin has the ability to bind a wide range of organic and inorganic ligands. The mechanism of ligands binding to the albumin molecule is determined by the presence of specific binding sites in the protein molecule. Some reactions of protein association with ligands are provided by electrostatic interactions, others are covalent in nature, causing chemical modifications of the side radicals of amino acids. The tertiary structure of this protein is determined by three domains [10]. It is assumed that subdomain IIA forms an important area for association with bilirubin and some dyes. There is an opinion that the main place of Methylene Blue binding to BSA is the subdomain IIA [11]. In addition, it is known that this protein is able to associate with anionic dyes of fluorescein family — fluorescein, erythrosine, eosin and Bengal pink [10]. Consequently, bovine serum albumin exhibits both anionic and cationic properties. If this fact is taken into account, then it becomes quite logical that direct interaction of albumin occurs with both polyphosphate and dye, thereby disrupting the aggregation of Methylene Blue. However, these interactions seem to be rather weak, since bovine serum albumin shifted the dye absorption spectra only at high concentration. Consequently, this protein has little effect on the metachromatic reaction.

Thus, based on the results obtained, it is assumed that with active accumulation of phosphorus in the volutin granules and a significant binding of metals to polyphosphates, which has been shown in our studies using the Ca^{2+} ions, the metachromatic reaction may not be observed. This occurs by shielding the anionic groups of the

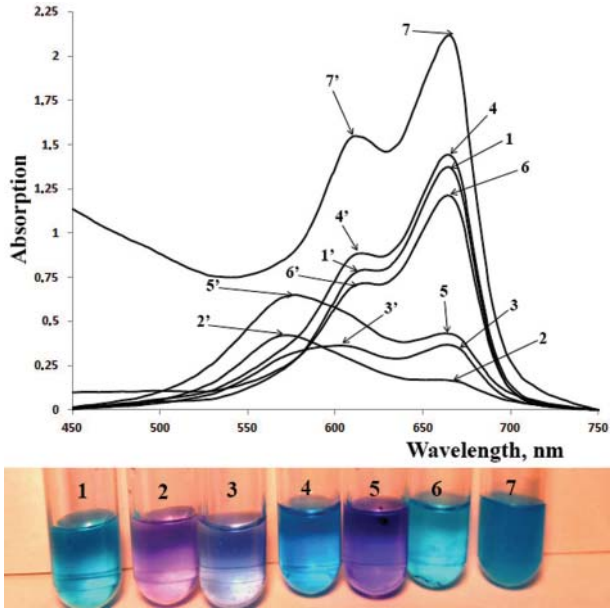

Fig. 5. **Absorption spectra (λ, nm) of Methylene Blue (MB, 0.05 mg/ml) and its complexes with polyphosphate with a chain length of 200 phosphoric residues ($PolyP_{200}$) and calcium chloride ($CaCl_2$) of different concentrations:**

1) MB (*1* — 664 nm, *1′* — 614 nm);
2) MB + 0.05 mg/ml of $PolyP_{200}$ (*2* — 664 nm, *2′* — 570 nm);
3) MB + 0.5 mg/ml of $PolyP_{200}$ (*3* — 664 nm, *3′* — 600 nm);
4) MB + 0.05 mg/ml of $PolyP_{200}$ + 0.1 mg/ml of $CaCl_2$ (*4* — 664 nm, *4′* — 614 nm);
5) MB + 0.5 mg/ml of $PolyP_{200}$ + 0.1 mg/ml of $CaCl_2$ (*5* — 664 nm, *5′* — 570 nm);
6) MB + 0.05 mg/ml of $PolyP_{200}$ + 1 mg/ml of $CaCl_2$ (*6* — 664 nm, *6′* — 614 nm);
7) MB + 0.5 mg/ml of $PolyP_{200}$ + 1 mg/ml of $CaCl_2$ (*7* — 664 nm, *7′* — 612 nm)

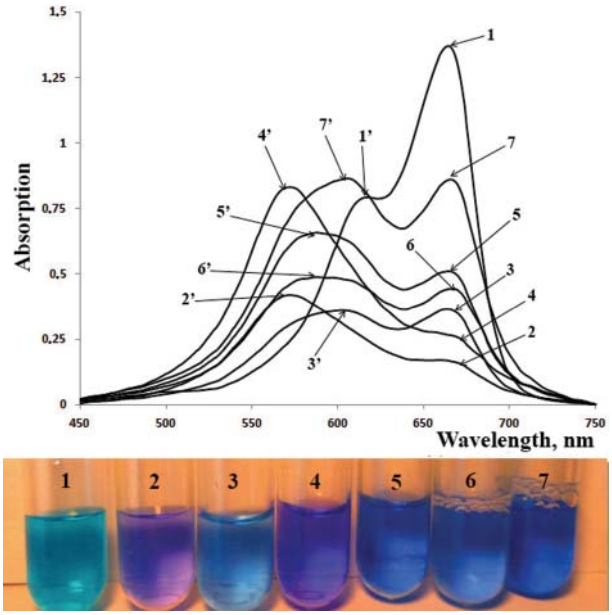

Fig. 6. **Absorption spectra (λ, nm) of Methylene Blue (MB, 0.05 mg/ml) and its complexes with polyphosphate with a chain length of 200 phosphoric residues ($PolyP_{200}$) and bovine serum albumin (BSA) of different concentrations:**

1) MB (*1* — 664 nm, *1′* — 614 nm);
2) MB + 0.05 mg/ml of $PolyP_{200}$ (*2* — 664 nm, *2′* — 570 nm);
3) MB + 0.5 mg/ml of $PolyP_{200}$ (*3* — 664 nm, *3′* — 600 nm);
4) MB + 0.05 mg/ml of $PolyP_{200}$ + 0.1 mg/ml of BSA (*4* — 664 nm, *4′* — 570 nm);
5) MB + 0.5 mg/ml of $PolyP_{200}$ + 0.1 mg/ml of BSA (*5* — 664 nm, *5′* — 600 nm);
6) MB + 0.05 mg/ml of $PolyP_{200}$ + 1 mg/ml of BSA (*6* — 664 nm, *6′* — 600 nm);
7) MB + 0.5 mg/ml of $PolyP_{200}$ + 1 mg/ml of BSA (*7* — 664 nm, *7′* — 605 nm)

polymer from the dye molecules. Consequently, the absence of metachromasy in the presence of the volutin granules themselves probably indicates the amount of absorbed phosphorus and metals by the microorganisms of active sludge. In this regard, the use of other cytochemical methods for the identification of polyphosphate granules is recommended, for example, staining with a extensively used elsewere fluorescent dye 4′, 6-diamidino-2-phenylindole (DAPI).

REFERENCES

1. *Serafim L.S., Lemos P.C., Levantesi C., Tandoi V., Santos H., Reis M.A.M.* Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J. Microbiol. Meth.* 2002, 51 (1), 1–18.
2. *Kulaev I. S., Vagabov V. M., Kulakovskaya T. V.* High-molecular inorganic polyphopshates: biochemistry, cell biology and biotechnology. *Moskva: Nauchnui Mir*, 2005, 216 p. (In Russian).
3. *Lorenz B., Schröder H. C.* Methods for investigation of inorganic polyphosphates and polyphosphate-metabolizing enzymes. *Inorganic Polyphosphates. Biochemistry, Biology, Biotechnology*. Schröder H.C., Muller W.E.G. (Eds *). Springer, Prog. Mol. Cell. Biol.*1999, P. 217–241.
4. *Pearse E.* Histochemistry, theoretical and applied. *Moscow: Foreign Lenguages Publishing House*, 1962, 962 p. (In Russian).
5. *Kulaev I.S.* Biochemistry of high-molecular polyphopshates. *Moscow University*, 1975, 247 p. (In Russian).
6. *Shirai M., Nagatsuka T., Tanaka M.* Interaction between dyes and polyelectrolytes. 2. Structural effect of polyanions on the methylene blue binding. *Micromol. Chem. Phys.* 1977, 178 (1), 37–36.
7. *Gromozova Ye. N., Grigoryev P. E, Kachur T. L., Voychuk S. I.* Influence of cosmophysical factors on a reaction of metachromasy in the volutine granules in Saccharomyces cerevisiae. Geophysical processes and biosphere. 2010, 9 (2), 67–76. (In Russian).
8. *Tewari K. K., Krishnan P. S.* Further studies on the metachromatic reaction of metaphosphate.

Arch. Biochem. Biophys. 1959, 82 (1), 99–106.
9. *Tagatsuki M., Yamaoka K. Metachromasy. V.* The effect of the chain length of polyphosphate on the metachromatic behavior of crystal violet and trypaflavine with emphasis on the binding curve and the spectra of bound dyes. *Bull. Chem. Soc. Jpn*. 1979, 52 (4), 1003–1009.
10. *Vlasova I. M., Kuleshova A. A., Vlasov A. A., Saletsky A. M.* Processes of molecular association and fluorescent characteristics of nanomarkers of fluorescein family in bovine serum albumin solution. *Moscow University Physics Bulletin*. 2013, 4, 39–44. (In Russian).
11. *Hu Y.-J., Liu Y., Zhao R.-M., Dong J.-X., Du S.-S.* Spectroscopic studies on the interaction between methylene blue and bovine serum albumin. *J. Photochem. Photobiol. A*. 2006, 179 (3) 324–329.

ВПЛИВ КОМПОНЕНТІВ СТІЧНИХ ВОД НА РЕАКЦІЮ МЕТАХРОМАЗІЇ ВОЛЮТИНОВИХ ГРАНУЛ *in vitro*

М. С. Харчук, О. М. Громозова

Інститут мікробіології і вірусології
ім. Д.К. Заболотного НАН України, Київ

E-mail: sithmcx@ukr.net

Мікроорганізми, що мають волютинові гранули, беруть активну участь у вилученні фосфору та важких металів зі стічних вод. Метою роботи було дослідити *in vitro* реакцію метахромазії в розчинах метиленового синього з поліфосфатами у поєднанні з іншими сполуками (протеїнами, вуглеводами, іонами металів). Встановлено, що вияв метахромазії залежить від концентрації та, меншою мірою, від довжини ланцюга поліфосфату. Додавання глюкози не призводило до змін ступеня вияву реакції метахромазії. Водночас іони кальцію і бичачий сироватковий альбумін залежно від концентрації стимулювали або інгібували метахроматичне забарвлення в тестових розчинах. Таким чином, значне накопичення поліфосфатів і катіонів металів, яке було показано нами на прикладі іонів Ca^{2+} мікроорганізмами активних мулів, може не завжди супроводжуватися вираженою реакцією метахромазії волютинових гранул. У зв'язку з цим доцільно використовувати інші цитохімічні методи для ідентифікації поліфосфатних гранул, зокрема фарбування 4′,6-діамідино-2-феніліндолом — DAPI.

Ключові слова: волютинові гранули, поліфосфат-акумулювальні організми, реакція метахромазії, очищення стічних вод.

ВЛИЯНИЕ КОМПОНЕНТОВ СТОЧНЫХ ВОД НА РЕАКЦИЮ МЕТАХРОМАЗИИ ВОЛЮТИНОВЫХ ГРАНУЛ *in vitro*

М. С. Харчук, Е. Н. Громозова

Институт микробиологии и вирусологии
им. Д. К. Заболотного НАН Украины, Киев

E-mail: sithmcx@ukr.net

Микроорганизмы, содержащие волютиновые гранулы, принимают активное участие в изъятии фосфора и тяжёлых металлов из сточных вод. Целью работы было изучение *in vitro* реакции метахромазии в растворах метиленового синего с полифосфатами в сочетании с другими соединениями (протеинами, углеводами, ионами металлов). Установлено, что проявление метахромазии зависит от концентрации и, в меньшей степени, от длины цепи полифосфата. Добавление глюкозы не приводило к изменению степени проявления реакции метахромазии. В то же время ионы кальция и бычий сывороточный альбумин в зависимости от концентрации стимулировали или ингибировали метахроматическую окраску растворов. Таким образом, значительное накопление полифосфатов и катионов металлов, показанное нами на примере ионов Ca^{2+} микроорганизмами активных илов, может не всегда сопровождаться выраженной реакцией метахромазии волютиновых гранул. В связи с этим рекомендуется использование других цитохимических методов для идентификации полифосфатных гранул, в частности, окрашивание 4′,6-диамидино-2-фенилиндолом — DAPI.

Ключевые слова: волютиновые гранулы, полифосфат-аккумулирующие организмы, реакция метахромазии, очистка сточных вод.