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A.G. Zhumina[1*], A.D. Orazbay[2], Y.N. Martynova[3], A.Zh. Abyurov[4], I.A. Safronova[5], M.P. Kulandin[6], D.M. Jexembayev[7]

[1, 2]*Karaganda National Research University named after academician E.A. Buketov, Karaganda, Kazakhstan;*
[3]*"Shubarkol Komir" JSC, Karaganda, Kazakhstan;*
[4]*Scientific and Engineering Research Center ERG LLP, Astana, Kazakhstan;*
[5, 6, 7]*Scientific and Engineering Research Center ERG LLP, Pavlodar, Kazakhstan*
[*]*Corresponding author: asbiol@list.ru*

Assessment of the cytotoxic activity of humates produced by "Shubarkol Komir" JSC

The development of new humic preparations from locally available raw materials is important for advancing the domestic industry of the Republic of Kazakhstan. In Central Kazakhstan, humic preparations such as potassium humate and sodium humate have been developed by "Shubarkol Komir" JSC based on weathered coal. To confirm their applicability, studies assessing their biological activity and safety are required. The purpose of this study is to evaluate the cytotoxic activity of potassium humate and a mixture of potassium humate and humate in different dilutions. The study materials included humates produced by "Shubarkol Komir" JSC, which were compared with a commercial preparation, ECO humate, using tests on *Saccharomyces cerevisiae* cells and *Artemia salina* nauplii. The test results showed that high concentrations of humic preparations (4 %) had an inhibitory effect on the foaming ability of yeast, which was lower than that of the comparison drug nystatin. Concentrations from 0.5 to 0.005 % did not inhibit *Saccharomyces cerevisiae* and activated the foaming ability. The results of the second experimental series demonstrated that both the humates produced by "Shubarkol Komir" JSC and the reference preparation were non-cytotoxic to *Artemia salina* larvae at all tested concentrations (0.5–0.005%), indicating their safety. Thus, the obtained data confirm the safety of using potassium humate and a mixture of potassium humate and sodium humate produced by "Shubarkol Komir" JSC in agriculture.

Keywords: humic preparations, potassium humate, sodium humate, cytotoxic activity, concentration, safety.

Introduction

The resources and effectiveness of organic fertilizers can be significantly increased through the use of humic fertilizers produced from various natural organic sources (peat, highly oxidized low-ash hard coal, brown coal, and oxidized brown coal, sapropel, organic industrial waste, etc.) containing large amounts of humic acids [1–4].

Humic preparations are complex biologically active substances containing humic acids and humates [5, 6], a balanced set of macro- and microelements, and microflora, which enhance the protective properties of plants and seedlings against a number of fungal and bacterial diseases [7–10].

It should be emphasized that humates are not a source of mineral nutrition and do not replace it, but only increase the coefficient of its utilization, therefore humate solutions can be used together with fertilizers, herbicides, and fungicides [11]. Thus, the use of humates in combination with mineral fertilizers and pesticides not only increases the yield of field crops and their efficiency, but also improves the quality of agricultural products [12].

There is also the problem of effective assimilation of mineral fertilizers, which is central to crop production. The complexity of solving this problem lays in the fact that potassium and nitrogen fertilizers, which are easily soluble in water, are easily washed out of the soil, while phosphorus fertilizers, on the contrary, are bound by calcium, magnesium, aluminum, and iron ions present in the soil into an inert form that is inaccessible to plants [13]. Only in the presence of humic substances does the efficiency of plant assimilation of all nutrients increase dramatically [14].

Humic preparations play an important role in increasing the biological activity of the soil, enriching seeds with macro- and microelements, and as growth stimulants [15]. Of particular interest are complex humic fertilizers, which have been used in agricultural production for a number of years.

The purpose of this study is to evaluate the cytotoxic activity of potassium humate, a mixture of potassium humate and sodium humate, and to determine safe concentrations for use on plants.

Experimental

The objects of the study were humate concentrates (4 %) provided by "Shubarkol Komir" JSC: potassium humate, a mixture of potassium humate and sodium humate (in a ratio of 2:1, respectively). Distilled water and a commercial sample of ESO humates served as controls in the studies. The tested humates were diluted to concentrations of 0.1 %, 0.01 %, 0.5 %, 0.05 %, and 0.005 %. Commercial ESO humate (manufactured by NTO EcoTek, Russia) was used according to the attached instructions — 0.1 %.

Assessment of the cytotoxic activity of humates

The test for cytotoxic activity of humates was carried out in two variants: on a culture of *Saccharomyces cerevisiae* cells and on larvae (nauplii) of *Artemia salina* crustaceans.

The first test consisted of assessing the rate of foam rise in a suspension of *Saccharomyces cerevisiae* yeast [16]. Dry active Pakmaya yeast (Turkey) was used. The antifungal drug Nystatin at a concentration of 5 mg/ml was used as a positive control sample. The drug was first ground in a mortar until a homogeneous mass was obtained, and then dissolved in water. A 10 % DMSO solution was used as a negative control.

Dough balls were prepared as follows: 0.2 g of glucose and 0.68 g of yeast were added to 9 ml of water. 1 ml of the test solution (samples of humates in different dilutions, DMSO, nystatin) was added to the resulting mixture. Three ml of the analyzed sample was transferred to measuring tubes, repeated three times for each tested sample (Fig. 1), and incubated in a thermostat at a temperature of 30ºC for 15 minutes.

After the time had elapsed, the volume of the foam formed and the rate of its rise were determined using the following formula:

$$v = V/t, \quad (1)$$

where v is the foam rise rate (ml/min); V is the foam volume (ml); t is the time (min).

An increase in foam rise velocity was considered a stimulating effect, and a decrease was considered an inhibiting effect compared to the control.

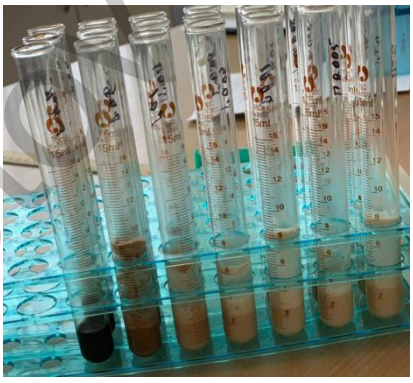

Figure 1. Experiments to study the lifting force of yeast in experiments with humic preparations

Artemia salina nauplii (Fig. 2) were used to conduct the second test for cytotoxic activity [17]. Artificial seawater was prepared for the crustacean culture (Tab. 1).

Table 1

Seawater composition

Component	Molecular weight	Added mass, g
NaCl	58.44	23.926
$Na_2 SO_4$	142.04	4.008
KCl	74.56	0.677

Continuation of Table 1

Component	Molecular weight	Added mass, g
Sodium bicarbonate ($NaHCO_3$)	84.00	0.196
Potassium bromide (KBr)	119.01	0.098
$H_3 BO_3$ (boric acid)	61.83	0.02
Sodium fluoride	41.99	0.003

To conduct the experiments, 200 mg of *Artemia salina* eggs were weighed and placed in 1 liter of artificial seawater. The eggs were kept aerated, under constant lighting and at a temperature of 25 °C for 2-3 days, until the larvae hatched (Fig. 2). After hatching, 20–40 larvae were collected using a Pasteur pipette and placed in a 2 ml cell of a laboratory plate. The number of live and dead larvae in each cell was counted. Ten micrometers of test solutions, positive and negative controls were added to the cells. The number of dead individuals was counted after 1, 4, and 24 hours. The mortality rate (P) was analyzed using the following formula:

$$P = (A — N — B) *100 \%/Z, \quad (2)$$

where A is the number of dead larvae after 24 hours; N is the number of dead larvae before the experiment; B is the average number of dead larvae in the control sample; Z is the total number of larvae.

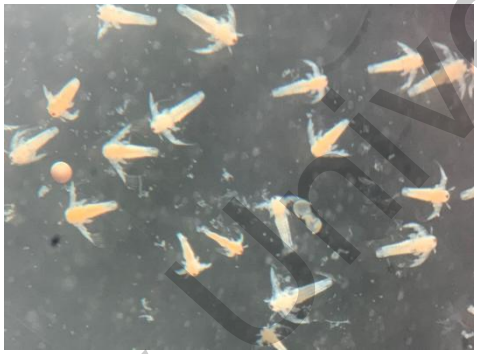

Figure 2. Nauplii of *Artemia salina*

The cytotoxic activity of humates was determined based on the number of dead larvae.

Statistical processing of data on cytotoxic activity was performed using ANOVA dispersion analysis with multiple comparisons according to Dunnet in the GraphPadPrizm 8.0 program.

Results and Discussion

The results of the assessment of the effect of humates on the buoyancy of *Saccharomyces cereviseae* showed that a mixture of sodium/potassium humates in a high concentration (4 %), had a significant inhibitory effect on the foaming activity *of Saccharomyces cereviseae*, which was significantly lower than the control values and the data obtained with the antifungal drug Nystatin. Concentrations ranging from 0.05 to 0.005 % showed a weak stimulating effect, which was significantly higher than the foam rise when using Nystatin, but significantly lower than the results obtained in the control. However, at concentrations of 0.1 % and 0.5 %, on the contrary, the mixture of humates has a significantly stimulating effect on yeast cells (Fig. 3).

When testing potassium humate, it was found that the concentrate of this preparation, like the mixture of humates, has an inhibitory effect on the foaming activity *of Saccharomyces cereviseae*. The foam rise indicators were significantly lower than the control and the indicators obtained when adding the preparation Nystatin. A concentration of 0.5 % potassium humate has a stimulating effect on yeast cells, while a further decrease in concentration shows no stimulating or inhibiting effect (Fig. 4).

Testing of the comparison preparation (humate ESO) also showed that its concentrate (10 %) has neither an inhibitory nor a stimulating effect on the foaming activity *of Saccharomyces cereviseae*. However, a decrease in concentration leads to inhibition of the foaming activity of yeast cells (Fig. 5).

Thus, statistical calculations showed that high concentrations of humic preparations produced by “Shubarkol Komir” JSC (4 %) had a depressing effect on the foaming ability of yeast, which was lower than that of the comparison preparation, the antifungal preparation nystatin. The humic preparation ESO at a con-

centration of 10 % did not show an inhibitory effect. However, humate concentrations of 4–10 % are not used in agriculture, as dilutions of at least 0.1-0.5 % are required.

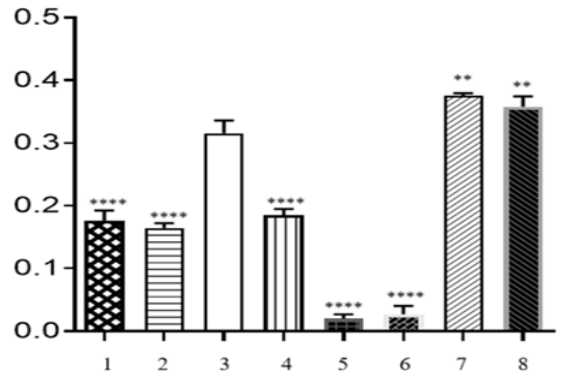

Figure 3. Effect of sodium/potassium humate mixture on the foaming activity *of Saccharomyces cereviseae*: one-way analysis of variance with multiple comparisons by Dunnet; ns — insignificant; **p>0.01; ****p>0.0001. The results are presented as mean ± standard deviation. Vertical axis — foam rise rate, ml/min; experimental variants: 1 — 0.005 %, 2 — 0.01 %, 3 — control, 4 — 0.05 %, 5 — 4 %, 6 — nystatin, 7 — 0.1 %, 8 — 0.5 %

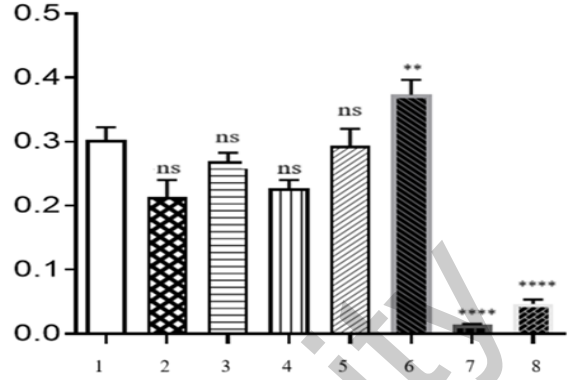

Figure 4. Effect of potassium humate on the foaming activity *of Saccharomyces cereviseae*: one-factor analysis of variance with multiple comparisons by Dunnet; ns — insignificant; **p>0.01; ****p>0.0001. Results are presented as mean ± standard deviation. Vertical axis — foam rise rate, ml/min; experimental variants: 1 — control, 2 — 0.005 %, 3 — 0.01 %, 4 — 0.05 %, 5 — 0.1 %, 6 — 0.5 %, 7 — 4 %, 8 — nystatin

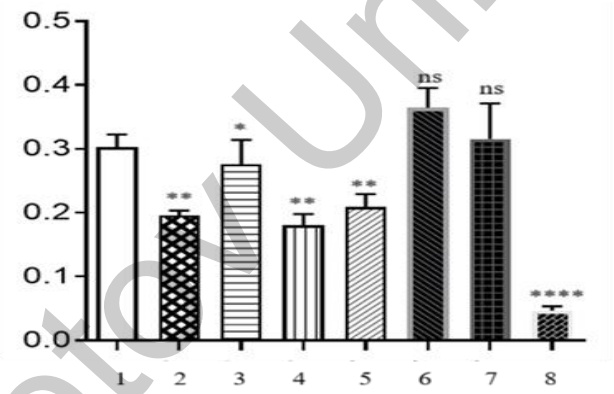

Figure 5. Effect of ESO humate on the foaming activity *of Saccharomyces cereviseae*: one-way analysis of variance with multiple comparisons by Dunnet; ns — insignificant;*p>0.05; **p>0.01; ****p>0.0001. The results are presented as mean ± standard deviation. Vertical axis — foam rise rate, ml/min; experimental variants: 1 — control, 2 — 0.005 %, 3 — 0.01 %, 4 — 0.05 %, 5 — 0.1 %, 6 — 0.5 %, 7 — 10 %, 8 — nystatin

Testing on *Artemia salina* brine shrimp at concentrations ranging from 0.5 to 0.005 % showed no inhibitory effect. According to the data obtained, mortality in samples treated with potassium humate at concentrations of 4 % and 0.5 % significantly exceeds that of the control sample. In addition, the difference in mortality between the control sample and the sample treated with humate at a concentration of 0.05 % is also statistically significant. However, despite this, all tested concentrations of potassium humate produced by "Shubarkol Komir" JSC are not cytotoxic to *Artemia salina* crustaceans, since the mortality of larval nauplii does not exceed 50 % (Fig. 6).

In experiments testing a mixture of potassium and sodium humate, mortality in samples treated with humate at concentrations of 4 % and 0.5 % significantly exceeded that of the control sample. In addition, the difference in mortality between the control sample and the sample treated with a mixture of potassium and sodium humates at a concentration of 0.05 % is also statistically significant. The mortality rate in the sample treated with a 4 % mixture of humates averages 72.2 %, which is much higher than 50 %, indicating that this

concentration is cytotoxic to *Artemia salina* larvae. However, as the concentration of the potassium and sodium humate mixture decreases, cytotoxicity decreases (Fig. 7).

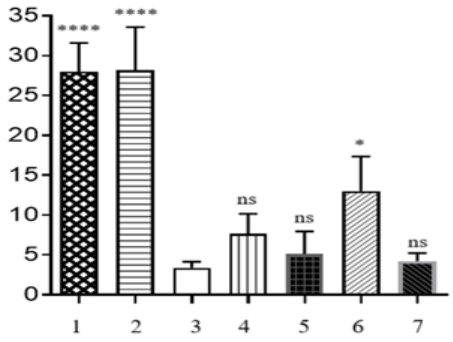

Figure 6. Effect of potassium humate produced by "Shubarkol Komir" JSC on the mortality of *Artemia salina* larvae: one-way analysis of variance with multiple comparisons by Dunnet; ns — insignificant; *p>0.05; ****p>0.0001. The results are presented as mean ± standard deviation. Vertical axis — larval mortality; experimental variants: 1 — 4 %, 2 — 0.5 %, 3 — control, 4 — 0.1 %, 5 — 0.005 %, 6 — 0.05 %, 7 — 0.01 %

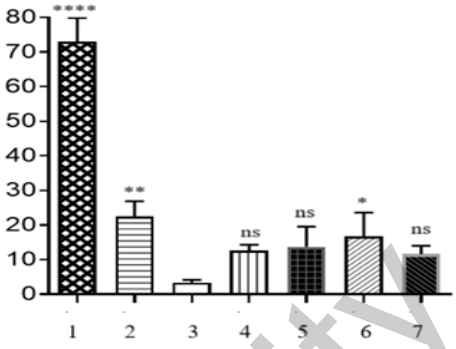

Figure 7. Effect of potassium and sodium humate mixture on *Artemia salina* larval mortality: one-way analysis of variance with Dunnet's multiple comparisons; ns — not significant; *p>0.05; **p>0.01; ****p>0.0001. Results are presented as mean ± standard deviation. Vertical axis — larval mortality; experimental variants: 1 — 4 %, 2 — 0.5 %, 3 — control, 4 — 0.1 %, 5 — 0.005 %, 6 — 0.05 %, 7 — 0.01 %

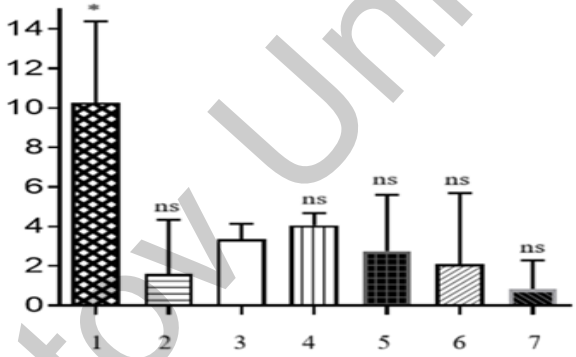

Figure 8. Effect of ECO humate on the mortality of *Artemia salina* larvae: one-way analysis of variance with multiple comparisons by Dunnet; ns — insignificant; *p>0.05. Results are presented as mean ± standard deviation. Vertical axis — larval mortality; experimental variants: 1 — 4 %, 2 — 0.5 %, 3 — control, 4 — 0.1 %, 5 — 0.005 %, 6 — 0.05 %, 7 — 0.01 %

According to the data obtained, the mortality in samples treated with ECO humate at a concentration of 10 % exceeds that of the control sample, but averages 10.2 %, indicating no cytotoxicity towards *Artemia salina* larvae. The results obtained in samples with all tested concentrations indicate that ECO humate is not cytotoxic (Fig. 8).

The data obtained allow us to conclude that humates produced by "Shubarkol Komir" JSC (potassium humate, a mixture of potassium and sodium humates) and the reference preparation (humate ESO) are not cytotoxic to *Artemia salina* larvae at all tested concentrations (from 0.5 to 0.005 %), i.e., the tested concentrations are safe for use.

Conclusion

The assessment of the toxicity of humic preparations on *Saccharomyces cereviseae* cells and *Artemia salina* larvae showed that all humates produced by "Shubarkol Komir" JSC are non-toxic, i.e., safe for use when diluted to concentrations of 0.5 % and below. All studied concentrations of potassium humate, mixtures of potassium and sodium humate, as well as the comparison preparation ESO, can be used in agriculture for plant treatment.

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Conflict of interest

The authors declare no conflict of interest.

Author contribution

The manuscript was written with contributions from all authors. All authors have approved the final version of the manuscript: **Zhumina A.G. —** conceptualization, investigation, writing draft; **Orazbay A.D. —** research, methodology; **Martynova Y.N. — investigation, statistical analysis; Abyurov A.Zh. — data curation, analysis; Safronova I.A. —** data collection**; Kulandin M.P., Jexembayev D.M. — humate production.**

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А.Г. Жумина, А.Д. Оразбай, Е.Н. Мартынова, А.Ж. Абюров, И.А. Сафронова, М.П. Куландин, Д.М. Жексембаев

«Шұбаркөл Көмір» АҚ өндірген гуматтардың цитотоксикалық белсенділігін бағалау

Жергілікті қолжетімді шикізаттан жаңа гуминдік препараттарды әзірлеу және жасау Қазақстан Республикасының отандық өнеркәсібін дамыту үшін өзекті. Орталық Қазақстанда «Шұбаркөл Көмір» АҚ-ның морыған көмірі негізінде калий гуматы және натрий гуматы сияқты гуминдік препараттар әзірленді. Оларды қолдану мүмкіндігін растау үшін биологиялық белсенділік пен қауіпсіздікті бағалау бойынша зерттеулер қажет. Зерттеудің мақсаты — әртүрлі сұйылтулардағы калий гуматының және калий гуматы мен гумат қоспасының цитотоксикалық белсенділігін бағалау. Зерттеулер «Шұбаркөл Көмір» АҚ өндірген гуматтарда Есо коммерциялық гуматымен салыстырғанда *Saccharomyces cerevisiae* жасушалары мен *Artemia salina* науплий шаян тәрізділерін пайдалана отырып жүргізілген сынақтарда жүргізілді. Тестілеу нәтижелері көрсеткендей, өндірілген гуминді препараттардың жоғары концентрациясы (4 %) ашытқылардың көбіктену қабілетіне тежегіш әсерін көрсетті, бұл нистатинді салыстыру көрсеткіштерінен төмен болды. 0,5-тен 0,005 %-ға дейінгі концентрациялар *Saccharomyces cerevisiae* депрессиясын көрсетпеді және көбіктену қабілетін белсендірді. Тәжірибелердің екінші сериясының нәтижелері «Шұбаркөл Көмір» АҚ өндірген гуматтардың және салыстыру препаратының барлық сыналған концентрацияларында (0,5-тен 0,005 %-ға дейін) *Artemia salina* шаян тәрізділердің дернәсілдеріне қатысты цитоуыттылығы жоқ екенін көрсетті, яғни сыналатын концентрациялар қолдану үшін қауіпсіз. Осылайша, алынған деректер «Шұбаркөл Көмір» АҚ ауыл шаруашылығында калий гуматын және калий гуматы мен натрий гуматының қоспасын қолданудың қауіпсіздігін растайды.

Кілт сөздер: гуминдік препараттар, калий гуматы, натрий гуматы, цитотоксикалық белсенділік, концентрация, қауіпсіздік

А.Г. Жумина, А.Д. Оразбай, Е.Н. Мартынова, А.Ж. Абюров, И.А. Сафронова, М.П. Куландин, Д.М. Жексембаев

Оценка цитотоксической активности гуматов производства АО «Шубарколь комир»

Разработка и создание новых гуминовых препаратов из местного доступного сырья является актуальным для развития отечественной промышленности Республики Казахстан. В Центральном Казахстане на основе выветрелых углей АО «Шубарколь комир» разработаны гуминовые препараты, такие как гумат калия и гумат натрия. Для подтверждения возможности их применения необходимы исследования по оценке биологической активности и безопасности. Цель настоящего исследования — оценить цитотоксическую активность гумата калия и смеси гумата калия и гумата натрия в разных разведениях. Исследования проводили на гуматах производства АО «Шубарколь комир» в сравнении с коммерческим гуматом ECO с использованием тестов на клетках *Saccharomyces cerevisiae* и науплий рачков *Artemia salina.* Результаты тестирования показали, что высокие концентрации гуминовых препаратов производства (4 %) показали угнетающее действие на пенообразовательную способность дрожжей, что было ниже показателей препарата сравнения — нистатином. Концентрации от 0,5 до 0,005 % не показали угнетения *Saccharomyces cerevisiae* и активировали пенообразовательную способность. Результаты второй серии опытов показали, что гуматы производства АО «Шубарколь комир» и препарат сравнения не обладают цитотоксичностью по отношению к личинкам рачков *Artemia salina* во всех протестированных концентрациях (от 0,5 до 0,005 %), то есть испытуемые концентрации являются безопасными для применения. Таким образом, полученные данные подтверждают безопасность применения гумата калия и смеси гумата калия и гумата натрия производства АО «Шубарколь комир» в сельском хозяйстве.

Ключевые слова: гуминовые препараты, гумат калия, гумат натрия, цитотоксическая активность, концентрация, безопасность

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Information about the authors

Zhumina Assel Galymovna — PhD, Dean of the Faculty of Biology and Geography, Karaganda National Research University named after academician E.A. Buketov, Kazakhstan; e-mail: asbiol@list.ru; ORCID: 0000-0002-0904-4726

Orazbay Aknur Daurenkyzy — Junior Researcher, Master of Biology, Faculty of Biology and Geography, Karaganda National Research University named after academician E.A. Buketov, Kazakhstan; e-mail: ladyasha@bk.ru; ORCID: 0009-0008-4469-7563

Martynova Yelena Nikolayevna — Research project Manager of "Shubarkol Komir" JSC, Karaganda, Kazakhstan; e-mail: Yelena.Martynova@erg.kz

Abyurov Arman Zhumagalievich — Engineer-technologist, Scientific and Engineering Research Center ERG LLP; Astana, Kazakhstan; e-mail: arman.Abyurov@erg.kz; ORCID: https://orcid.org/0000-0002-0336-0111

Safronova Inessa Aleksandrovna — Head of Coal Chemistry Laboratory, Scientific and Engineering Research Center ERG LLP; Pavlodar, Kazakhstan; e-mail: inessa.Safronova@erg.kz

Kulandin Mikhail Petrovich — Technologist, Scientific and Engineering Research Center ERG LLP; Pavlodar, Kazakhstan; e-mail: Mikhail.Kulandin@erg.kz

Jexembayev Doskhan Muratkhanovich — Project Manager, Scientific and Engineering Research Center ERG LLP; Pavlodar, Kazakhstan; e-mail: doskhan.Jexembayev@gmail.com; ORCID: https://orcid.org/0009-0004-4765-7701