BIOCHAR ENHANCES ANTIBIOTIC-RESISTANT GENES REMOVAL FROM AQUEOUS ECOSYSTEMS

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ABSTRACT

Pollution of aquatic ecosystems with antibiotic-resistant genes originating from human medicine and veterinary is an urgent problem due to the potential threaten to public health. Antibiotic-resistant genes enter surface waters and wastewater through vertical and horizontal water runoff. At the same time, heavy metals and biogenic substances often presented in aqueous ecosystems often exacerbate the problem since the drive the horizontal transfer of antibiotic-resistance genes. To solve the problem of purification of waters from antibiotic-resistant genes, the adsorbing agents, such as biochar, might be used. In this work, we studied the effect of biochar on the dynamics of the content of tetracycline-resistant genes in a liquid LB medium with a microbial community transferred to the medium from compost. The following additives were used - Cu (600 $\mu g*l^{-1}$), Cd (130 $\mu g*l^{-1}$), Ni (70 $\mu g*l^{-1}$), Fe (1500 $\mu g*l^{-1}$), humic acids (6%), oxytetracycline (300 mg/l). Real-time PCR revealed the absence of the *tet*(O) gene both in all variants with and without biochar. The highest excesses over control were found for the *tet*(M) and *tet*(C) genes. The introduction of biochar made it possible to reduce the content of antibiotic-resistant genes in all samples with different additives. Thus, in the variant with Cd, the content of the *tet*(A), *tet*(B), *tet*(C) и *tet*(S) gene was eliminated. The *tet*(A), *tet*(E) и *tet*(S) genes were completely absent in the sample with antibiotic.

Keywords: antibiotic resistance, antibiotic-resistant genes, biochar, aqueous ecosystems, wastewater.

INTRODUCTION

The spread of antibiotic-resistant bacteria and antibiotic-resistant genes in aquatic ecosystems is a serious problem due to the use of huge amounts of antibacterial drugs not only in medicine but also in agriculture [1]. Since antibiotics are poorly absorbed in the gastrointestinal tract, animal manure contains residual amounts of antibiotics, as well as antibiotic-resistant microflora [2]. Antibiotic-resistant genes enter open water bodies along with horizontal and vertical water runoff from pastures, as well as wastewater discharges from agricultural enterprises. The problem is exacerbated by the fact that antibiotics do not decompose completely in domestic wastewater treatment plants due to their low hydrophilicity and structural stability [3].

An increase in the number of antibiotic-resistant genes in water bodies is facilitated by horizontal gene transfer from one bacterium to another. In addition, heavy metals and biocides often found in water bodies and wastewater can promote horizontal transfer of antibiotic resistance genes, increasing selective pressure on microorganisms

[4]. As a result, pathogenic resistant bacteria such as vancomycin-resistant *Staphylococcus aureus* (VRSA), methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Acinetobacter baumannii* (MRAB), multidrug-resistant *Pseudomonas aeruginosa* (MRPA) have been found in aquatic ecosystems [3].

A serious problem regarding the spread of antibiotic-resistant genes is also biogenic pollution of water bodies coming from wastewater and their subsequent eutrophication, since an increase in organic compounds in the aquatic environment creates favorable physicochemical and nutritional conditions for the reproduction of resistant bacteria and the horizontal transfer of antibiotic-resistant genes [3,4].

Thus, due to a direct threat to public health, an urgent task is to remove resistant bacteria and antibiotic resistance genes from the aquatic environment. Existing wastewater treatment methods (coagulation, chlorination, ozonation, UV irradiation, combined disinfection, membrane filtration) reduce the number of antibiotic resistance genes, but compared to the natural environment, the resistance gene contamination profile often remains high. In addition, after treatment, there was a decrease in the number of antibiotic-resistant genes with a simultaneous significant increase in the number of broad-host-range plasmids [5].

Recent studies have shown that the use of sorbent agents such as biochar is effective in purifying water from antibiotic-resistant genes [6]. The suppression of proliferation and spread of resistance genes in the aquatic environment occurs mainly due to adsorption mechanisms. Biochar can reduce the bioavailability of heavy metals and biocides, which helps to reduce the selective pressure on microorganisms and reduce the spread of antibiotic resistance. In addition, biochar can influence changes in the structure of the microbial community, reducing the proportion of resistant bacteria and, accordingly, the number of antibiotic-resistant genes [7]. Such effects have been noted in many studies for plant-based biochar. From the point of view of the substrate for the production of biochar, dung and manure are also promising - in their unprocessed form they pose a threat in terms of the presence of residual quantities of antibiotics and antibiotic-resistant genes in them, which can lead to the spread of antibiotic resistance. However, there is little information on the use of biochar, made from manure and dung, in relation to the purification of water bodies from antibiotic resistance genes.

In this work, we assessed the effectiveness of using biochar based on chicken manure to remove tetracycline-resistant genes from the aquatic environment. Additional factors in the study were water pollution by oxytetracycline, metals and biogenic elements.

MATERIALS & METHODS

To prepare an aqueous extract with a microbial community, mature compost prepared on the basis of bedding chicken manure mixed with sawdust in a ratio of 33% (w:w) was used. The compost was mixed with water in a ratio of 1:10 and vortexed for 1 hour. Next, 1 ml of the prepared extract was added to 50 ml of liquid LB medium. Additionally, following additives - Cu (600 $\mu g*l^{-1}$), Cd (130 $\mu g*l^{-1}$), Ni (70 $\mu g*l^{-1}$), Fe (1500 $\mu g*l^{-1}$), humic acids (6%), antibiotic oxytetracycline (300 $mg*l^{-1}$) were added to flasks. Variants with the same additives were also prepared, but with the introduction of biochar into the LB medium at a dose of 15%. The variant without additives with neutral pH was used as a control. The prepared flasks were incubated for 3 days at 28°C

and 100 rpm. On the 3rd day of incubation, samples were taken for further DNA extraction.

Extraction of DNA from samples was carried out using the FastDNA Spin Kit for Soil (MP Bio, Germany) according to the manufacturer's instructions. DNA purification was carried out using the QIAquick PCR Purification Kit (Qiagen, Germany). Detection of *tet*(A), *tet*(B), *tet*(C), *tet*(E), *tet*(M), *tet*(S) and *tet*(X) genes was carried out by real–time PCR [8]. In detail, the PCR products of *tet*(A), *tet*(B), *tet*(C), *tet*(E), *tet*(M), *tet*(S) and *tet*(X) were first cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). Then, the plasmids carrying *tet*(A), *tet*(B), *tet*(C), *tet*(E), *tet*(M), *tet*(S) and *tet*(X)were extracted and purified using a PureLink Quick Plasmid Miniprep Kit (Invitrogen Corporation, Carlsbad, CA) [9]. The master mix reaction mixture (25 µl) contained the following components: DNA template – 1 µl, forward and reverse primers (10 µM) – 0.5 µl each, dNTPs (10 µM) – 2.5 µl, 10x Buffer with SYBR Green – 2.5 µl, $MgCl_2$ (25 µM) – 2.5 µl, SynTaq polymerase (5 U µl-1) – 0.2 µl and ddH_2O – 15.3 µl. Amplification was performed on a BioRad CFX–96 cycler using the following temperature program: primary denaturation at 95°C for 5 min, then 39 three–step cycles at 62-60°C for 45 seconds, at 95°C for 15 seconds, and at 72°C for 30 seconds.

RESULTS AND DISCUSSION

In the control sample on the 3rd day of incubation, the studied tetracycline-resistant genes were found, with the exception of the *tet*(O) gene (Figure 1), since the microbial community of the extract was obtained from the compost of poultry receiving antibiotics. The *tet*(B) and *tet*(X) genes ($5.32*10^7$ and $1.66*10^8$ copies*ml^{-1}, respectively) were characterized by the highest number of gene copies, while the *tet*(E) gene was characterized by the smallest number ($2.29*10^1$ copies*ml^{-1}). The optical density in the control sample on the 3rd day of incubation was 0.085 (Figure 2).

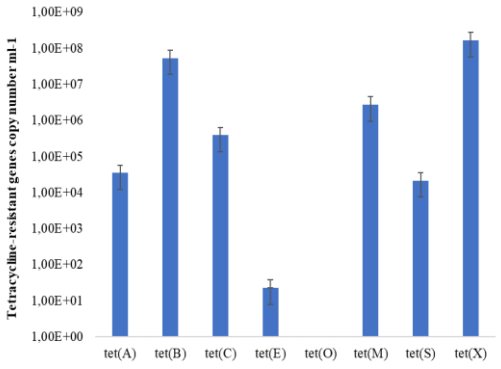

Figure 1. Content of tetracycline-resistant genes in the control sample, copy number*ml^{-1}

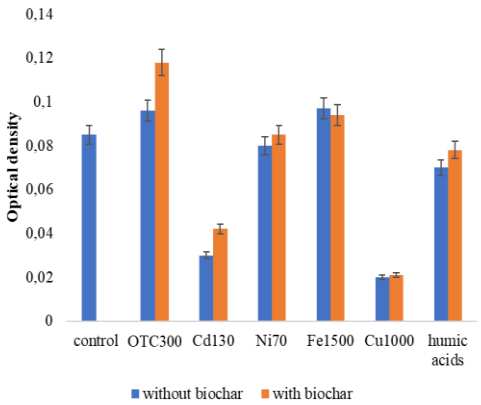

Figure 2. Optical density of samples with/without biochar

In samples containing metals, on the 3rd day of incubation, the *tet*(O) gene was also not detected, both in variants with biochar and without it (Figure 3 a-d). The greatest excesses over control were found for the *tet*(C) and *tet*(S) genes (2935% and 1232%, respectively), and the lowest excess was found for the *tet*(B) gene (0.03%). The increase

in the number of some antibiotic-resistant genes could be caused by additional selection of resistant microorganisms and stimulation of horizontal transfer in the presence of metals. The introduction of biochar in some cases allowed to reduce the content of antibiotic-resistant genes in the microbial community. Thus, in the variant with Cu, the *tet*(E) gene was eliminated; in the variant with Ni, the *tet*(A) and *tet*(X) genes completely disappeared; in the variant with Fe, the *tet*(B) and *tet*(S) genes disappeared.

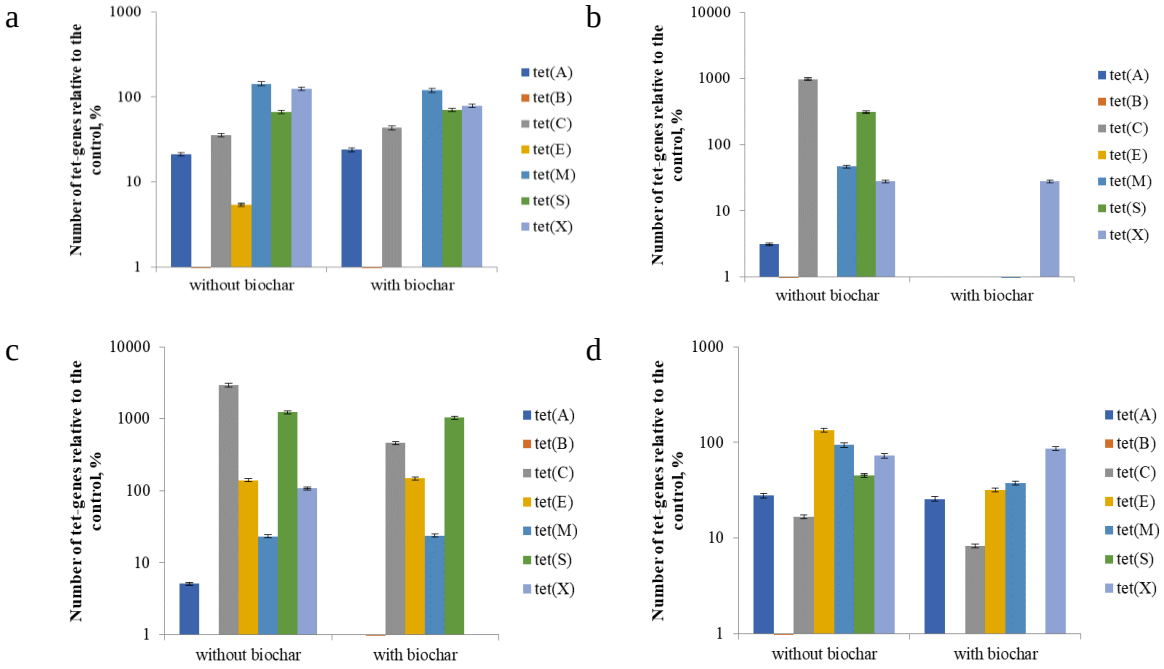

Figure 3. Content of tetracycline-resistant genes in samples with the addition of metalls (a – Cu, b – Cd, c – Ni, d – Fe), copy number*ml^{-1}

The introduction of biochar most effectively affected the reduction of antibiotic-resistant genes in the variant with Cd. Elimination of the *tet*(A), *tet*(B), *tet*(C), and *tet*(S) genes was found in this sample. At the same time, it is interesting to compare the data on the content of antibiotic-resistant genes with the data on the optical density of the samples. So, in the variants with Ni and Fe, the optical density was at the control level (0.08–0.085 and 0.097–0.094, respectively) and did not depend on the introduction of biochar (Figure 2). In the variants with Cd and Cu, the optical density was significantly lower compared to the control, while in the variant with Cd, an increase in the growth of microorganisms in the presence of biochar was observed. Probably, in this case, biochar contributed to more favorable conditions for the microbial community, creating additional niches for microorganisms in the form of pores [10]. The decrease in the content of antibiotic-resistant genes was due to the adsorption properties of biochar and a decrease in the bioavailability of metals. A number of works also indicate a high adsorption capacity of biochar in the treatment of wastewater contaminated with metals [11]. Thus, Wu et al. demonstrated the removal of 78.99%, 82.99%, 31.189%, 48.381%, 52.031%, 33.834% and 43.84% of the *tet*(W), *tet*(M), *sul*1, *sul*2, *oxa*1, *qnr*S and *intI*1 genes, respectively, from metal-contaminated wastewater using biochar [7].

As in the samples with Cd and Ni, in the sample with OTC, the greatest excess of the number of antibiotic-resistant genes over the control was observed, since the

presence of the antibiotic contributed to the formation of resistance in the microbial community and its spread through horizontal gene transfer (Figure 4) [12]. The *tet*(A) and *tet*(M) genes were characterized by the greatest excess over the control (5757% and 30676%, respectively), while the lowest excess over control was found for the *tet*(B), *tet*(E), and *tet*(S) genes (7.7%, 20% and 6%, respectively).

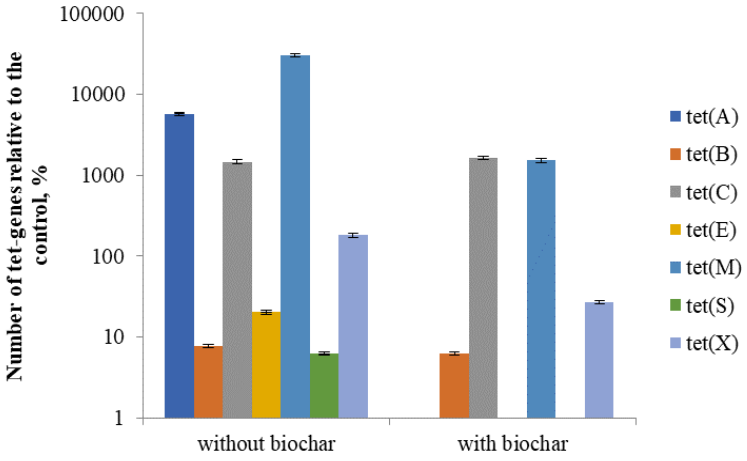

Figure 4. Content of tetracycline-resistant genes in samples with the addition of OTC, copy number*ml^{-1}

In general, the introduction of biochar had a negative effect on the content of antibiotic resistance genes. Thus, a reduction in the number of *tet*(M) and *tet*(X) genes and elimination of the *tet*(A), *tet*(E), and *tet*(S) genes was found. The content of the *tet*(B) and *tet*(C) genes in the variants with biochar remained similar to their content in the variants without it. The optical density of the sample with OTC without biochar correlated with that in the control, while in the presence of biochar, stimulation of microorganism growth was observed (Figure 2). In the works of a number of authors, a positive effect of the use of biochar on the content of antibiotic-resistant genes in wastewater treatment was also noted [6,13]. In the literature, this is explained by the fact that, on the one hand, biochar can change the structure of the microbial community, while reducing the proportion of resistant species, and on the other hand, it can directly absorb residual amounts of antibiotics themselves, reducing their bioavailability and, as a result, the spread of antibiotic resistance genes. In addition, biochar can absorb and destroy extracellular DNA carrying resistance genes and mobile genetic elements, thereby slowing down horizontal transfer [14].

In the sample with humic acids, the *tet*(O) gene was not found, as in the samples with other additives, the remaining genes were present in an amount exceeding that in the control sample. The excess content of antibiotic-resistant genes is probably due to the fact that the additional presence of organic substances is a source of nutrition for resistant microorganisms, promoting their reproduction and stimulating horizontal transfer[13]. The *tet*(C), *tet*(E), and *tet*(M) genes) were found with the greatest excess over the control (125%, 68% and 116%, respectively).

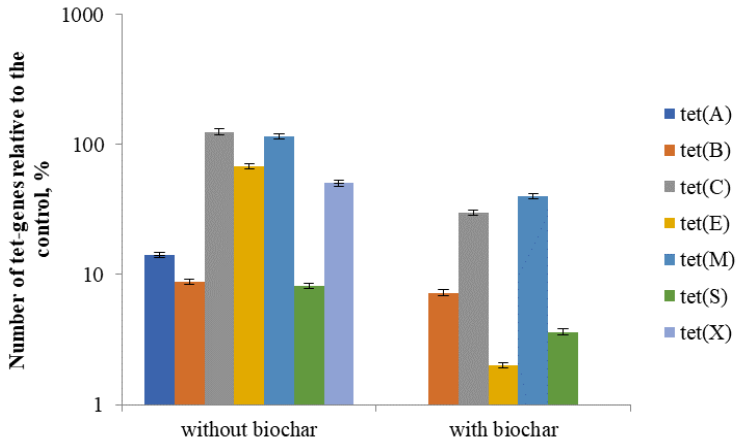

Figure 2. Content of tetracycline-resistant genes in samples with the addition of humic acids, copy number*ml^{-1}

The introduction of biochar contributed to a decrease in the content of the *tet*(C), *tet*(E), *tet*(M), and *tet*(S) genes, while elimination of the *tet*(A) and *tet*(X) genes was observed. The optical density of this sample in the presence of biochar was slightly higher than without it and correlated with the control sample. Biogenic compounds often found in wastewater increase the problem of antibiotic resistance, as they create favorable nutritional and physico-chemical conditions for resistant bacteria. In the literature it is demonstrated the high efficiency of using biochar to treat water contaminated with biogenic compounds [3,4,6].

CONCLUSION

In this study, the efficiency of using biochar based on chicken manure to remove tetracycline-resistant genes from aquatic environments contaminated with metals (Cu, Cd, Ni, Fe), antibiotic (oxytetracycline) and biogenic compounds (humic acids) was evaluated. It was demonstrated that metals, antibiotic and organic contamination of water led to excess of the content of antibiotic-resistant genes *tet*(A), *tet*(B), *tet*(C), *tet*(E), *tet*(M), *tet*(S) and *tet*(X) as compared with uncontaminated control. Addition of 15% biochar to the aquatic samples led to the reduction or complete disappearance of antibiotic-resistant genes' content. In samples containing metals, biochar showed the highest efficiency in the variant with Cd, where four *tet*-genes (*tet*(A), *tet*(B), *tet*(C), and *tet*(S)) were completely eliminated. In the sample containing oxytetracycline, the introduction of biochar eliminated three *tet*-genes: *tet*(A), *tet*(E), and *tet*(S). In the sample contaminated with humic acids, two *tet*-genes, *tet*(A) and *tet*(X), completely disappeared in the presence of biochar.

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