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Polycyclic aromatic hydrocarbons, antibiotic resistance genes, toxicity in the exposed to anthropogenic pressure soils of the Southern Russia

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ABSTRACT

The influence of anthropogenic pollution, particularly with polycyclic aromatic hydrocarbons (PAHs) on soil toxicity and spread of antibiotic resistance genes (ARGs) is extremely important nowadays. We studied 20 soil samples from a technogenically polluted site, municipal solid wastes (MSW) landfills, and rural settlements in the southwestern part of the Rostov Region of Russia. A close correlation was established between the results of biosensor testing for integral toxicity, the content of genes for the biodegradation of hydrocarbons, and the concentration of PAHs in soils. The relation between the quantitative content of ARGs and the qualitative and quantitative composition of PAHs has not been registered. Soils subjected to different types of the anthropogenic pressure differed in PAHs composition. The technogenic soils are the most polluted ones. These soils are enriched with 5 ring PAHs and carry the maximum variety of assayed ARGs, despite the fact that they do not receive household or medical waste.

1. Introduction

Despite the recognition of the global importance of the environmental anthropogenic pollution problem, the influx of pollutants into various natural environments continues. One of the most polluted compartments under severe anthropogenic pressure is the soil used for storage and disposal of municipal and industrial waste. Typical examples of such areas are municipal solid waste (MSW) landfills and industrial sludge collectors (Duan et al., 2008; Pérez-Leblic et al., 2012; Melnyk et al., 2015; Swati et al., 2017; Petrovic et al., 2018; Koshlaf et al., 2019).

Landfills are currently the most common solid waste management strategy worldwide, especially in developing countries (Eggen et al., 2010). Various municipal waste, household chemicals, pharmaceuticals, natural substances and products of their processing, as well as toxic substances are utilized at MSW landfills. The filtrate formed in the waste layer is a combination of various pollutants, including organic compounds, ammonia nitrogen, heavy metals, polycyclic aromatic hydrocarbons (PAHs). Through rainfall, such a filtrate can enter open water reservoirs along with groundwater (Clarke et al., 2015; Wang et al., 2015; Yi et al., 2017).

Industrial waste is one of the most difficult to recycle. It poses a serious threat to the environment, since most of such waste is extremely toxic, and substances in the liquid physical form easily penetrate soil and migrate to adjacent environments. Such soils contain a wide range of different organic and inorganic pollutants. One of the most dangerous pollutants is PAHs, which not only have significant toxicity, but are also capable of affecting the genetic apparatus of living cells, exhibiting mutagenic and carcinogenic properties.

The wide spread of antibiotic resistance genes (ARG) and antibiotic resistant bacteria (ARB) is currently considered another serious environmental problem that threatens human and animal health (Berendonk et al., 1988). Taking into account the mutagenic potential of PAHs and their ability to accelerate the adaptive evolution of bacteria (Kim et al., 2015; Pérez-Pantoja et al., 2013; Akkaya et al., 2019; Sazykin et al., 2019a), as well as the abundance of microbial environment facilitating

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the horizontal transfer of ARGs, there is a high probability of contamination of anthropogenically modified soil and adjacent surface, ground waters and soils with ARBs and ARGs (Sazykin et al., 2019c; Song et al., 2016).

To combat these negative phenomena effectively, understanding of the pollution nature is necessary. For adequate assessment of such contamination, a comparison of data obtained during the identification of pollutants (e.g., PAHs), soil toxicity biotesting, and PCR analysis of metagenomic DNA for detection of clinically important ARGs, hydrocarbon biotransformation genes, etc. is necessary.

Currently, environmental monitoring includes analysis by means of different biosensors, including those based on whole-cell bacterial lux biosensors (Woutersen et al., 2011; Elad and Belkin, 2017; Gui et al., 2017). It is very convenient to use such test systems because of their high sensitivity, rapidity, and cost-effectiveness, which allows them to be used for initial screening of a large data array in order to isolate various groups for further chemical analysis (Tsybulskii and Sazykina 2010; Sazykina et al., 2012; Sazykin et al., 2015; Behera et al., 2018).

Several MSW landfills in the southwestern part of the Rostov Region were selected as objects of study. These included functioning landfills, a recently closed one and one closed and reclaimed several decades ago. To assess soil pollution with industrial waste, the Lake Atamanskoye, located in the left-bank floodplain of the Seversky Donets River, was examined. The lake was used to collect wastewater from textile and chemical enterprises located in Kamensk-Shakhtinsky from the 1950s to the mid-1990s (Privalenko et al., 2000). By now, the lake has degraded and dried up, and contaminated bottom sediments were exposed to the surface (Konstantinova et al., 2020).

This work aimed to assess the effect of PAHs soil contamination on the distribution of clinically relevant ARGs in soil microbiome and on soil toxicity determined by the biotesting method.

2. Materials and methods

2.1. Sampling of soils

The soils were sampled during May–June 2017, from the studied territory from the depth of 0–20 cm by the envelope method (GOST 17.4.4.02-84, 2000; Directive document 2.1.7.730-99, 1999), thoroughly mixed, distributed into Falcon plastic tubes (50 mL) and stored at −20 °C for preparation of extracts, total DNA isolation and PAHs assay. Soil sampling was carried out at MSW landfills in Rostov-on-Don and the Rostov Region, on the outskirts of rural settlements and in the area of technogenically polluted dried up lake Atamanskoye (Fig. 1).

2.2. Chemicals and reagents

Chemicals and reagents used in experimental work can be found in Supplementary Table 1.

2.3. Assessment of soil pollution using biosensors

A battery of whole-cell bacterial lux biosensors was used to assess the toxicity of samples of the studied soils. The natural strain *Vibrio aquamarinus* VKPM B-11245 and *E. coli* MG1655 (pXen7-lux) were used to determine the integral toxicity of the samples (Sazykin et al., 2014, 2016). Inducible luminescent bacterial biosensors *E. coli* MG1655 (pRecA-lux) and *E. coli* MG1655 (pColD-lux) were used to detect genotoxicants (Biran et al., 2009; Sazykin et al., 2016; Abilev et al., 2020). To determine genotoxicity based on metabolic activation, the S9 rat liver microsomal enzyme fraction was used (Moltox, USA). *E. coli* MG1655 (pKatG-lux) and *E. coli* MG1655 (pSoxS-lux) biosensors were used to assess oxidative stress inducing substances in the cell (Zavilgelsky et al., 2007; Sazykin et al., 2016; Manukhov et al., 2008).

Bioluminescent strains were obtained by transformation of *E. coli* MG1655 by hybrid plasmids pXen7, pRecA-lux, pColD-lux, pKatG-lux, pSoxS-lux. The *Photorhabdus luminescens* gene cassette *luxCDABE* under the control of *Plac, PrecA, pColD, PkatG, PsoxS* promoters, respectively, was used in these biosensors. The plasmids were constructed on the basis of pBR322 vector and contain a selective ampicillin resistance marker.

Bacterial strains were cultivated in Luria-Bertani medium (LB medium, containing tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L), supplemented with 100 μg of ampicillin/ml, at 37 °C overnight. Then the main cultures were inoculated to optical densities (OD_{590}) of 0.025 from an overnight culture and cultivated at 37 °C for 2.5 h to OD_{590} of approximately 0.2 determined by FLUOstar Omega microplate reader (BMG Labtech, Germany). Cells were used immediately for stress induction tests. The assessment of soil pollution was carried out using bacterial biosensors.

Luminescence was measured with Luminoskan Ascent microplate luminometer (Thermo Fisher Scientific, USA) in three independent replications.

2.4. PAHs extraction and determination

PAHs were extracted from the soils using alkaline saponification (Directive document 52.10.556-95, 2002; Sushkova et al., 2019). A 1.0 g portion of the prepared soil was put in a pear-shaped flask for rotary

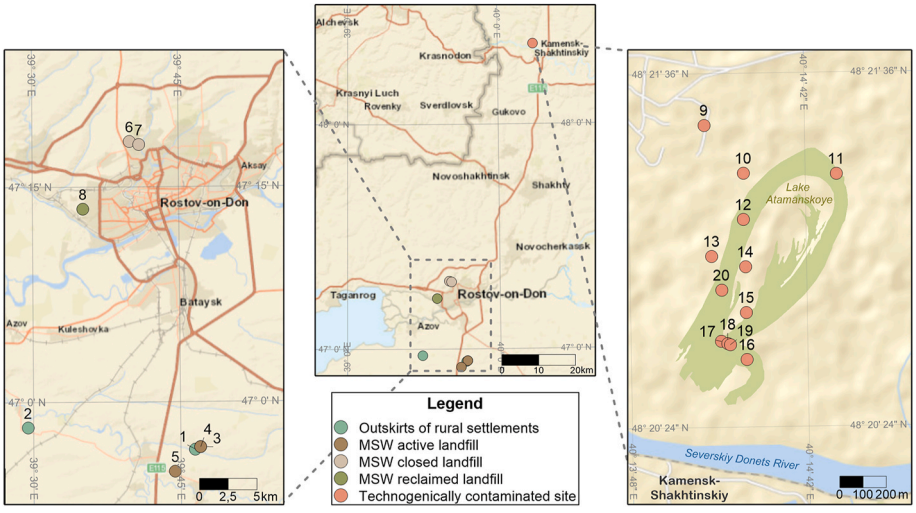

Fig. 1. Simplified map of study area and location of sampling sites.: 1–2 - outskirts of rural settlements; 3–8 - MSW landfills (3–5 - active, 6–7 - recently closed, 8 - closed and reclaimed several decades ago); 9–20 - technogenically contaminated sites.

evaporator; 20 mL of 2% KOH solution in ethanol was added, and the mixture was refluxed on a water bath for 3 h. The saponificaiton of lipids and gummy soil components occurred during the refluxing, which increased the recovery of PAHs and reduced the amount of coextracted substances in the extract. The supernatant was decanted into an Erlenmeyer flask, and 15 mL of n-hexane and 5 mL of distilled water were added for the better separation of the layers. The mixture was shaken on a rotary shaker for 10 min and transferred into a dividing funnel. The hexane layer was poured into a separate vessel. The residue in the flask was extracted twice more in a similar way. The combined hexane extract was washed with distilled water to neutral pH (using litmus as an indicator), transferred into a dark vessel with a close lid, and desiccated by adding 5 g of anhydrous Na_2SO_4. After exposure at +5 °C for 8 h, the desiccated extract was decanted into a dry round-bottomed flask and evaporated to dry on a rotary evaporator at a bath temperature of 40 °C. The dry residue was dissolved in 1 mL of acetonitrile.

The content of PAHs in the extracts was quantified by 1260 Infinity (Agilent, USA) high performance liquid chromatograph (HPLC) equipped with a fluorescence detector following the ISO 13859:2014 requirements. The HPLC system was fitted with reversed phase column Hypersil BDS C18 (125 × 4.6 mm, 5 μm) and a mixture of acetonitrile and ultrapure water as the mobile phase. Thirteen PAHs were measured in the present study: naphthalene (NAP), biphenyl (BIP), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR),benzo [a]anthracene (BaA), benzo [b]fluoranthene (BbF), benzo [k]fluoranthene (BkF), benzo [a]pyrene (BaP), dibenzo [a,h]anthracene (DBA), and benzo [g,h,i]perylene (BghiP). Compounds were identified by comparing the retention times to that of the analytical standard samples.

Quality control of every HPLC detection was performed according Agilent Application Solution (ISO 13877–2005). Individual standard solutions were purchased from the Sigma-Aldrich (Merch). A calibration standard of PAHs mixture was injected after every six samples to correct for drift in retention time within a run. The certified reference materials and calibration curves were used for calculation of the limits of detection (LODs) and limits of quantification (LOQs) have been presented previously (Minkina et al., 2020). The total PAHs value means average from 3 analytic replications. Statistical significance of the differences among means was determined by least significant difference (LSD) test. Differences were considered not significant at values of $P > 0.05$.

2.5. Isolation of total DNA from soil samples

Total metagenomic DNA was isolated from the soil, which was investigated to detect ARGs and hydrocarbon degrading genes using PCR amplification method.

To isolate DNA, a sample of 0.2 g of frozen soil was placed in a 2 mL tube with a screw cap, to which glass (0.5 mm and 1.0 mm) and ceramic (1.0 mm and 2.0 mm) beads were added. Then, 350 μL of guanidine solution (guanidine HCl 240 mM; sodium phosphate buffer 200 mM; pH 7.0) and 350 μL of SDS (2%) - Tris (500 mM) solution, as well as 400 μL of phenol-chloroform mixture were added to each tube. The mixture was shaken on a Mixer Mills MM400 (Retsch, Germany) for 15 min at the frequency of 30 Hz, then centrifuged for 7 min at 14,000 g. The aqueous phase was collected, 400 μl of chloroform was added to it. It was centrifuged in the same way as at the previous stage, then the aqueous phase was taken and 500 μl of isopropyl alcohol was added to it. It was kept in a refrigerator for about 15 min then centrifuged for 7 min at 14,000 g. The precipitate was washed 2 times with 70% ethanol and dissolved in 1X TE buffer. Additional purification of the obtained preparations was carried out on spin columns from the NucleoSpin Soil kit (Macherey-Nagel, Germany).

2.6. Analysis of antibiotic resistance genes

Reagent kits for PCR amplification with endpoint detection (NPF Litekh, Russia) were used to determine resistance to carbapenems (*VIM, NDM, OXA-48* genes), cephalosporins (*CTX-M, MecA* genes), glycopeptides (*VanA* and *VanB* genes), erythromycin (*ErmB* genes), tetracycline (*TetM/TetO* genes) (Supplementary Table 1).

Qualitative characterization of the bacteriocenosis resistome of the studied soil samples was carried out by PCR in accordance with the manufacturer's instructions. Amplicon electrophoresis was performed in horizontal 1.2% agarose gel (TBE buffer) for 1 h at the 5 V/cm voltage. EF gels were stained with ethidium bromide and visualized using the Gel Doc XR gel documentation system (Bio-Rad).

2.7. Analysis of hydrocarbon biotransformation genes

The following genes of the initial steps of hydrocarbon degradation were selected to evaluate the hydrocarbon-oxidizing potential of the microbial community: alkane monooxygenase *alkB* gene, *nahAc* naphthalene dioxygenase gene, *CYP 153A* cytochrome P450 gene, *bphA* biphenyl dioxygenase α-subunit gene. Primers were selected based on nucleotide sequences of the corresponding annotated genes publicly available in the NCBI database.[1]

The nucleotide sequence of primers selected for the genes of the initial stages of hydrocarbon degradation is shown in Supplementary Table 2.

Qualitative determination of the genes of mono- and dioxygenases of hydrocarbons in samples of total soil DNA was carried out by end-point PCR on a T-100 amplifier (Bio-Rad). The reaction mixture with a total volume of 25 μl contained 1X PCR-Buffer-B, deoxynucleoside triphosphates – 0.2 mM of each, $MgCl_2$ – 2.5 mM, SynTaq DNA polymerase with inhibiting enzyme activity antibodies – 1.25 units. (Synthol, Russia), primers – 0.4 μM each and 10 ng of metagenomic DNA. Amplification modes were optimized for each pair of primers (Supplementary Table 3). Amplicons were detected by their fragment size using horizontal gel electrophoresis.

2.8. Semi-quantitative determination of the amount of ARG and genes of hydrocarbon degradation initial stages

The semi-quantitative assessment of PCR amplicons was achieved by digital analysis of PCR-electrophoresis gels using a freely available image analysis software-ImageJ. To do this, the intensity of the target bands glow (weighted average number of pixels) was determined on a gel image with the Roi manager tool. Then, relative to bands with a known DNA concentration, the amount of DNA in the desired bands was determined, taking into account the size of the amplicon and the number of amplification cycles. The number of ARGs and genes of the initial stages of hydrocarbon degradation was determined in correlation relative to the number of 16 S rRNA genes.

2.9. Data analysis and statistics

The induction factor, (F_i) was defined as the relation of luminescence intensity of a lux-biosensor suspension, containing tested sample (L_c), to the luminescence intensity of a lux-biosensor control suspension (L_k): $F_i = L_c/L_k$.

When the degree of luminescence induction is evaluated in environmental samples, it should be noted that many of the substances included in their composition, can enhance and suppress bacterial bioluminescence, influencing the bacterial luciferase enzyme that can cause artifacts. Isogenic *E. coli* strain MG1655 (pXen7), whose lux operon is under the control of a constitutive promoter, was used to correct artifacts associated with changes in luciferase activity.

Therefore, besides, the induction factor coefficient of luminescence suppression *(K)* was determined: $K = l_c/l_k$.Where l_c – luminescence

[1] https://www.ncbi.nlm.nih.gov/.

intensity suspension lux-strain with constitutive promoter in the presence of the test compound; I_k – luminescence intensity control suspension lux-strain with constitutive promoter.

The correct values of the induction factor (I), served as a measure of toxicity for the genetically engineered *E. coli* strains, were calculated using the formula: $I = F_i/K$, where F_i – induction factor, K – coefficient of luminescence suppression.

All the experiments were carried out three times independently. The differences from control were considered statistically significant at $p < 0.05$. The detected toxic effect for the genetically engineered *E. coli* strains according to the I values was evaluated as follows: weak toxicity ($I < 2$), moderate toxicity ($2 \leq I \leq 10$), and strong toxicity ($I > 10$).

Strong toxic influence of the studied toxicant on *V. aquamarinus* is evaluated according to the inhibition of its bioluminescence for 30-min exposition period. Toxicity index (T) was used to determine integral toxicity of the samples calculated according to the formula:

$$T = 100(I_k - I_c) / I_c$$

where I_c and I_k are the intensity of bacteria luminescence in proof and control samples, respectively, at fixed exposition time of the studied solution with test object.

The following toxicity classes were estimated using *V. aquamarinus:* admissible ($T < 20$), toxic ($20 \leq T < 50$), and highly toxic ($T \geq 50$).

Pollution levels of PAHs in the soil samples were evaluated using the pollution index (*PI*), calculated as the ratio of total PAHs content in the studied soil sample compared to the regional background value. The natural soil from specially protected natural reservation of the Persian Protected Steppe was considered as a background. The total PAHs content in the soil of the long-term fallow regime ordinary chernozem was 250 μg/kg (Popileshko et al., 2019). The following soil contamination classes were identified according to *PI* values (Kowalska et al., 2018) as: absent (< 1), low (1–2), moderate (2–3), strong (3–5), and very strong (> 5) contamination.

Four PAH diagnostic ratios were calculated to determine the appropriate source of pollution (i.e. petrogenic or pyrogenic). According to Li et al. (2006), PAHs of petrogenic origin were characterized by the ratio ANT/(ANT + PHE) < 0.1, whereas the ratio ANT/(ANT + PHE) > 0.1 corresponded to pyrogenic nature. The ratio FLT/(FLT + PYR) < 0.4 indicated petrogenic sources, while that between 0.4 and 0.5 related to petroleum burning, and the ratio > 0.5 implied incomplete combustion of coal, wood or grass (Yunker et al., 2002; Rocher et al., 2004). The FLU/(FLU + PYR) ratio is used to differentiate diesel (> 0.5) and gasoline (< 0.5) emissions (Ravindra et al., 2008).

At these ratios, PAHs have similar molecular weights and physiochemical properties, thus assumed to behave similarly in the environment. In addition, the ratio of low molecular (LMW) PAHs to high molecular (HMW) PAHs were calculated. LMW PAHs include NAP, BIP, FLU, PHE, and ANT, while HMW PAHs comprise FLT, PYR, BaA, BbF, BkF, BaP, DBA, and BghiP. The ratio LMW/HMW > 1 indicated petrogenic sources of PAHs, whereas the ratio LMW/HMW < 1 corresponds to pyrogenic sources (Soclo et al., 2000; Rocher et al., 2004).

The software package STATISTICA 12 (StatSoft, USA) was used for statistical analysis of the data, and Grapher 11 (Golden Software, USA) was used for data visualization. Cluster analysis (CA) was used to classify the studied soils depending on the composition and sources of PAHs. Soils were combined using the hierarchical CA according to Ward's amalgamation method and Euclidean distances as distance metric. Generalized CA according k-Means algorithm were used to analyze the structure of the classified groups. ANOVA was performed to determine the contribution of each variable to the resulting model. Spearman rank correlation eco-efficiencies (r) were calculated to identify relationships among the PAH content and the luminescent response of biosensors. Principal component analysis (PCA) with varimax rotation with Kaiser normalization was used to analyze the relationship between concentrations of 13 PAHs and ARGs and hydrocarbon biotransformation genes in all samples studied in order to detect possible effects of soil contamination. Only components with eigen values above 1.0 were taken into account, following the Kaiser criteria.

3. Results

3.1. Soil bioassay using bacterial lux biosensors

The assessment of integral toxicity of the samples showed that the soils of settlements have a low level of toxicity. Among the soils sampled at solid waste landfills, two samples showed a high level and four samples displayed average levels of toxicity. In 6 out of 12 samples of technogenically contaminated soils a high degree of toxicity was detected and in two samples the permissible degree of toxicity was noted, 4 samples had an average level of toxicity (Fig. 2).

Promutagenic and mutagenic substances were detected using bacterial lux-biosensors *E. coli* MG1655 (pRecA-lux) and *E. coli* MG1655 (pColD-lux) in all three types of soils. The number of samples contaminated with promutagens slightly exceeded the number of samples contaminated with direct mutagens detected by both biosensors. 50% of the samples contained substances of both classes.

The revealed degree of genotoxic contamination of the studied soils in the overwhelming majority of contaminated samples was insignificant. Interestingly, 2 samples from solid waste landfills and 2 samples from a technogenically contaminated site, but not the soils of settlements had the lowest level of genotoxicity.

The average genotoxic effect due to promutagens was demonstrated by soil sample no. 8 (reclaimed solid waste landfill closed several decades ago). This sample was the most genotoxic of all the studied. The results obtained using both biosensors *E. coli* MG1655 (pRecA-lux) and *E. coli* MG1655 (pColD-lux) complement each other and require combined use to characterize genotoxicity.

In 14 out of 20 studied samples, the *E. coli* MG1655 biosensor (pKatG-lux) which reacts to the presence of peroxides showed an average level of toxicity (I ranges from 2.07 to 3.24). The maximum induction coefficient (I = 3.24) was recorded in a technogenically contaminated soil sample.

An insignificant amount of peroxides was found in two soil samples from solid waste landfills and three samples of technogenically contaminated soils. *E. coli* MG 1655 (pSoxS-lux), a biosensor that reacts to redox compounds causing superoxide radical anion and nitric oxide generation showed an average value of prooxidant activity only in 2 samples of technogenically contaminated soils (I = 2.02 and I = 2.75).

In the remaining samples toxicity was insignificant. Thus, the prooxidant activity of the studied anthropogenically contaminated soils is determined, first of all, by the presence of peroxides and only to a small extent by redox compounds and nitrogen oxide generators.

According to the sum of the toxic effects identified by all biosensors,

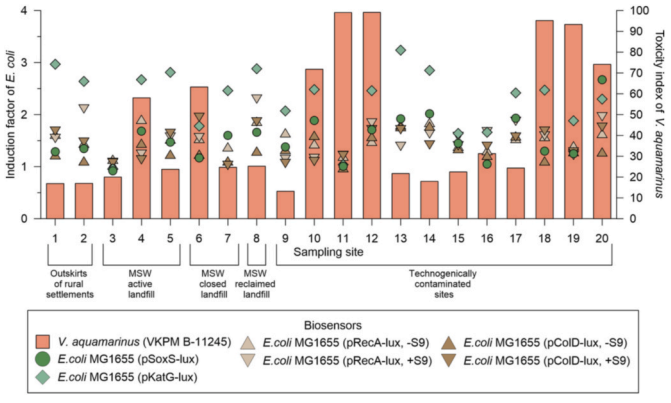

Fig. 2. Variation in toxicity of the studied soil samples depending on the land use.

two soil samples from technogenically contaminated sites should be noted (No. 12 and 17). One of these samples had the highest integral toxicity, and the other had a low value of integral toxicity in the middle range.

3.2. Contamination of the studied soils by PAH

The concentrations of 13 individual PAHs in soils with different degrees of anthropogenic influence are shown in Supplementary Table 4. The concentration of ΣPAHs in the soils of the studied area ranged from 146.6 to 4786.7 μg/kg with a mean of 1727.8 μg/kg, which was seven times higher than in background natural soils. The average total PAH content in the soils increases in the following order: outskirts of rural settlements (195.1 μg/kg) < MSW reclaimed landfills (342.2 μg/kg) < MSW active landfills (468.7 μg/kg) < technogenically contaminated soils of Lake Atamanskoye (2248.0 μg/kg) < MSW closed landfills (2720.7 μg/kg). The PAH content in the studied soils correlates with the results obtained for the soils undergoing a long-term effect of emissions from the Novocherkassk thermal electric power plant (382.4–1604.2 μg/kg) (Sazykin et al., 2019b).

Compared to the natural chernozem from the Persian Protected Steppe natural reservation, soils from outskirts of rural settlements were characterized as uncontaminated (PI < 1). The level of soil contamination of MSW active landfills varied from negligible (sites 3 and 5) to strong (site 4). The soils of MSW closed landfills were characterized by a strong and very strong contamination, which indicated a long-term intensive accumulation of PAHs, at the same time, reclamation of landfills reduced pollution to a low level (PI = 1.37). The level of PAH contamination of the soils from the Lake Atamanskoye varied greatly. The soils of the sites farthest from the former lake (sites 9–10) were characterized by low pollution (PI 1.4 and 1.7), while in its peripheral (sites No. 14–16) and central parts (sites No. 11–13 and 17–20) there was a strong (PI 3.53–4.53) and very strong pollution (PI 6.52–19.15), respectively.

In total, individual compounds, in ascending order of a mean concentration, were: ANT < BIP < FLU < NAP < BaA < BaP < BkF < FLT < PYR < DBA < PHE < BghiP < BbF. The content of individual PAH varied considerably, reflecting the diversity of sources between areas with different land use. On the average, PHE and ANT dominated in the soils of settlements (61.5 and 39.2 μg/kg, respectively), as well as in the soils of MSW reclaimed landfills (98.2 and 77.2 μg/kg, respectively). The high proportion of LMW PAHs indicated either the petrogenic origin of PAHs due to fuel or petroleum spills (Cao et al., 2017), or biomass low-temperature pyrolysis (Bao et al., 2018). The soils of MSW active landfills were mainly characterized by the accumulation of BghiP (68.9 μg/kg), PHE (66.5 μg/kg), PYR (63.3 μg/kg), and FLT (62.9 μg/kg), while the soils of MSW closed landfills accumulated BghiP (652.0 μg/kg), FLT (438.1 μg/kg), and BbF (336.9 μg/kg) much more intensively. An increase in HMW PAHs indicate the duration and intensity of the anthropogenic load, since they have a tendency toward more intensive sorption in soils due to high lipophilicity and bioaccumulation (Tsibart and Gennadiev, 2013; Jiang et al., 2016; Cao et al., 2017). Technogenically contaminated soils on average, differed from all others by the predominance of BbF (423.6 μg/kg), DBA (283.0 μg/kg), and BghiP (263.8 μg/kg).

3.3. PAH source identification

The sources of PAHs in the studied soil samples were determined using the diagnostic ratios (Fig. 3). The LMW/HMW PAHs ratio in almost all soils was less than 1, which indicated the pyrogenic origin of PAHs. The exception was the soils of site 1, located on the outskirts of the settlement, and site 5, from the side dump of the MSW active landfill, which were characterized by petrogenic input of PAHs. Similar results were obtained using the ANT/(ANT + PHE) ratio, according to which PAHs in all studied soils are of pyrogenic origin. At the same time, the FLT/(FLT + PYR) ratio varied from 0.26 to 0.54, respectively, three groups of sources are distinguished: petrogenic (30% of samples), combustion of petroleum (25% of samples) and combustion of biomass (45% of samples). The ratio FLU/(FLU + PYR) < 0.5 showed that the input of PAHs during the petroleum combustion was associated with the operation of vehicles with gasoline engines. Therefore, taking into account all the results obtained, it can be concluded that in the soils on the outskirts of settlements and from MSW active landfills, PAHs are predominantly of a mixed origin. Presumably PAHs enter the soils on outskirts of settlements due to the influence of vehicles, including gasoline emissions and spills of oil products, and into the soils of MSW active landfills as a result of the plastic combustion and the presence of various organic solvents in the waste. PAHs enter the soils of MSW closed landfills mainly due to regular landscape fires, in which the grass growing on the territory is burned. Various petrogenic sources of PAHs are characteristic of technogenically contaminated soils. Probably, the sources of PAHs in these soils were contaminated bottom sediments, which became the parent rock, as well as natural burning of vegetation.

The studied soils differed not only in the content of PAHs, but also in the composition of the compounds (Fig. 4). The presence of PAHs in the soils of the studied territories is due to the properties of the soil or any other sorbent substrate, as well as the intensity of the impact of the source and the nature of its origin (Sushkova et al., 2018). Thus, it is advisable to combine the soils of the monitoring sites into classes according to the composition of PAHs.

Hierarchical dendrogram showing clustering of studied soils according to Ward's method, using the Euclidean distance, is presented in Fig. 5. The results of CA allowed us to detect three separate clusters based on the PAH composition. ANOVA for the PAH groups showed significant differences ($p < 0.005$).

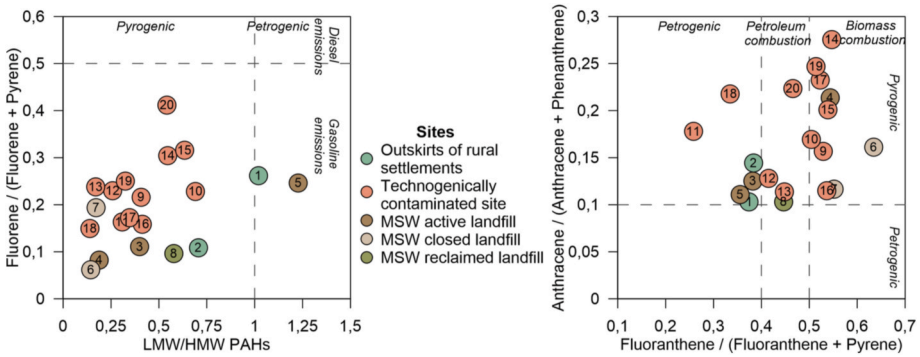

Fig. 3. PAH diagnostic ratios in the studied soil samples. The low molecular weight (LMW) PAHs refers to naphthalene, biphenyl, fluorene, phenanthrene, and anthracene; the high molecular weight (HMW) PAHs refers to fluoranthene, pyrene, benzo [a]anthracene, benzo [b]fluoranthene, benzo [k]fluoranthene, benzo [a] pyrene, dibenzo [a,h]anthracene, and benzo [g,h,i]perylene.

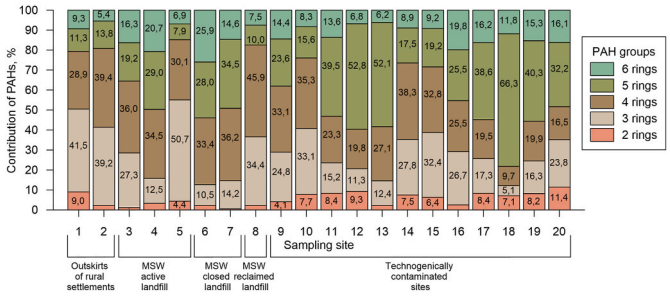

Fig. 4. Percentage contribution of the PAHs groups to the total PAH concentration in the studied soil samples. PAH groups include compounds with 2 rings: naphthalene and biphenyl; with 3 rings: fluorene, phenanthrene, and anthracene; with 4 rings: fluoranthene, pyrene, and benzo [a]anthracene; with 5 rings: benzo [b]fluoranthene, benzo [k]fluoranthene, benzo [a]pyrene, and dibenzo [a,h]anthracene; and with 6 rings - benzo [g,h,i]perylene.

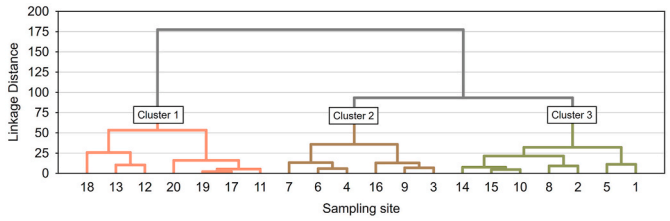

Fig. 5. Hierarchical dendrogram showing clustering of studied soils by PAH composition according to Ward's method, using Eucledian distance.

The soils of cluster 1 were the most polluted soils from the Lake Atamanskoye area. These relatively young soils formed on technogenic bottom sediments, which for a long-time accumulated PAHs in the composition of industrial effluents. A characteristic feature of this cluster was the dominance of 5-ring compounds. The average contribution of PAH groups decreased in the following order: 5 rings (45.0%) > 4 rings (18.1%) > 3 rings (14.8%) ≈ 6 rings (13.3%) > 2 rings (8.8%). Such a composition profile of PAH is typical for landfills of industrial effluents (Salihoglu et al., 2010; Hamid et al., 2016; Suman et al., 2016; Basavaiah et al., 2017).

Cluster 2 combined mainly soils of active and closed MSW landfills and slightly contaminated soils from the Lake Atamanskoye area. These soils showed the maximum proportion of 4 and 5-ring compounds, which is most typical for landfills (Salihoglu et al., 2010; Daso et al., 2016; García-Martínez et al., 2018). Most likely, the group composition of PAHs reflects the input of compounds due to the biomass combustion. The PAH groups, in descending order of mean contribution, were: 4 rings (32.3%) ≈ 5 rings (30.3%) >3 rings (18.3%) ≈ 6 rings (16.8%) > 2 rings (2.3%).

Cluster 3 united mainly slightly polluted soils of the outskirts of rural settlements, periphery of active and reclaimed MSW landfills, as well as forested soils of the Lake Atamanskoye area. The soils of this cluster were characterized by an increased proportion of 3 and 4-ring compounds in the composition of PAHs as compared to the soils of the other two clusters. The PAH groups, in descending order of average contribution were 3 rings (37.0%),4 rings (35.8%), 5 rings (13.6%), 6 rings (7.9%), 2 rings (5.6%). Such a composition profile of PAH is typical for areas subject to minimal technogenic pressure (Wang et al., 2019; Suman et al., 2016; Tsibart and Gennadiev, 2013).

3.4. Relationship between the PAH content in the soils and the luminescent response of biosensors

Results of correlation analysis showed statistically significant relationship ($p < 0.05$) between the PAH content and the luminescent response of the biosensor strain *V. aquamarinus* VKPM B-11245. The closest relationship was observed for BghiP and BkF (r was 0.719 and 0.716, respectively). Noticeable positive correlations ($0.5 < r < 0.7$) were observed with the rest of the individual compounds and ΣPAHs. There were no significant correlations between response of inducible *E. coli* MG1655 biosensor strains and the content of PAHs in soils samples; dependencies appear only at the level of individual clusters.

Cluster 1 revealed significant ($p < 0.05$) close positive correlation between the biosensor *V. aquamarinus* VKPM B-11245 and BIP ($r = 0.821$), as well as close negative correlations ($r = -0.821$) between *E. coli* MG1655 (pKatG-lux) biosensor and ANT, and biosensor *E. coli* MG1655 (pColD-lux) without metabolic activation and BghiP. In less contaminated soils of cluster 2, strong positive correlations ($r = 0.943$) were noted between biosensor strain *V. aquamarinus* VKPM B-11245 and content of ANT and BghiP. Also, the positive response of the biosensor *V. aquamarinus* VKPM B-11245 increases with an increase in the concentration of PHE, FLT, PYR, BaP, and ΣPAHs in soils ($r > 0.8$). Close positive correlations ($r = 0.829$) were observed in pairs *E. coli* MG1655 (pRecA-lux) biosensor with metabolic activation and NAP, and *E. coli* MG1655 (pColD-lux) biosensor without metabolic activation and BIP. In the soils of cluster 3, characterized by a minimal technogenic load, a close positive correlation ($r > 0.8$) was noted between the response of the *E. coli* MG1655 (pColD-lux) biosensor without metabolic activation and the contents of NAP, FLU, PHE, FLT, PYR, BaA, BghiP, and ΣPAHs in soils.

It is interesting to note that, depending on the qualitative and quantitative composition of hydrocarbon contamination, the degree of influence of individual hydrocarbons on the effect-specific bacterial biosensor that responds to DNA-tropic substances can vary.

3.5. Identification of antibiotic resistance genes in soil samples

Ten clinically significant ARG were determined in 20 soil samples taken in the territories of MSW landfills (6 samples), technogenically contaminated site (12 samples) and on the outskirts of rural settlements (2 samples). The carbapenemase genes – *VIM, NDM* and *OXA-48*, cephalosporin resistance genes – *CTX-M* and *MecA*, glycopeptides – *VanA* and *VanB*, erythromycins – *ErmB* and tetracycline – *TetM/TetO* were determined. The presence of six ARGs – *VIM, NDM, CTX-M, VanA, VanB*, and *TetM/TetO* was established in the studied soils. The *OXA-48, MecA*, and *ErmB* genes were not detected (Supplementary Table 5).

One of the most dangerous ARGs evaluated in this study were carbapenemases, since they have high catalytic activity, and a wide range of substrate specificity, including almost all classes of beta-lactam antibiotics (Ulyashova et al., 2010; Djenadi et al., 2018). Genes encoding carbapenemases, *VIM*, and *NDM* were determined in 4 of the 20 samples studied. These were samples from the outskirts of rural settlements (*NDM* gene – sites 1 and 2), as well as a technogenically polluted site (*VIM* – site 10 and *NDM* – site 16).

Cephalosporin resistance genes (*CTX-M*) were detected in 5 of the 20 samples studied (Nos. 1, 2, 9, 12, 18). *CTX-M* were present in all soil samples from settlements, in 25% of technogenically contaminated samples and were completely absent in the soils of solid waste landfills.

Also, in 6 of the 20 samples studied (Nos. 4, 6, 10, 15, 17, 18), genes encoding glycopeptide resistance (*VanA, VanB*) were found. Tetracyclines are broad-spectrum antibiotics that are most commonly used in veterinary medicine. Tetracycline resistance genes (*TetM/TetO*) were present in 2 test samples (nos. 4 and 16).

Resistance to glycopeptides and tetracyclines was found in technogenically polluted soils and soils of solid waste landfills, but not in soils of settlements.

It should be noted that in soil samples from rural settlements, genes determining resistance to carbapenems (*NDM*) and cephalosporins (*CTX-M*) were detected simultaneously.

The PCA results were obtained by employing varimax rotation with Kaiser normalization and presented graphically in Fig. 6a. Three

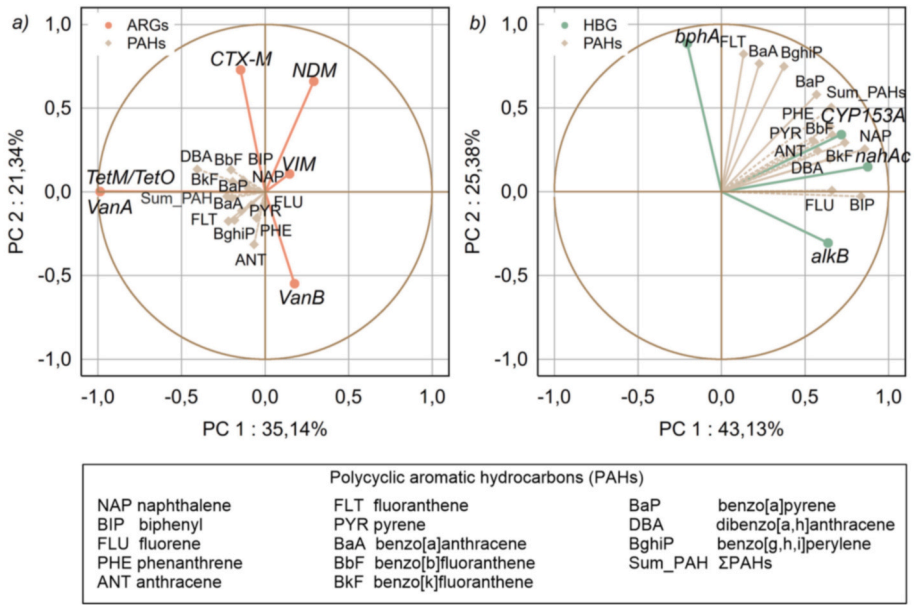

Fig. 6. Factor loadings plots of PAHs and (a) antibiotic resistance genes (ARGs) and (b) hydrocarbon biotransformation genes (HBG) on main principal components. The length of the arrows approximates the variance of the variables, whereas the angles among them estimate their correlations.

principal components (PC1, PC2 and PC3) were extracted with eigen values > 1, accounting for cumulative variance of 74.4% for the soil samples. The first component (PC1) describes 35.1% of the total variance and shows strong negative loadings for *VanA* (−0.987) and *TetM/TetO* (−0.987). The second component (PC2) explains 21.3% of the total variance and exhibits strong positive loading for *CTX-M* (0.729) and moderate loading for *NDM* (0.660), as well as moderate negative loading for *VanB* (−0.549). The third component PC3, explaining 17.9% of the total variance, is strongly associated with *VIM* (0.907). These data were plotted for the two main factors, ARGs and PAH concentrations (Fig. 6a), and show that resistance to various types of antibiotics develops depending on the specific environmental conditions, while the content and composition of PAHs are probably of secondary importance.

3.6. Identification of genes of the initial stages of hydrocarbon biotransformation using PCR

Soil samples were examined for the presence of genes *alkB* (alkane monooxygenase), *nahAc* (large subunit of naphthalene dioxygenase), *bphA* (large subunit of biphenyldioxygenase), and cytochrome P450 of the *CYP153* family, which determine alkane hydroxylase activity. The *alkB* and *nahAc* genes were found in the soils of solid waste landfills and technogenically contaminated sites, but not in the soils of settlements (Supplementary Table 6). The *bphA* gene was detected only in one soil sample of the solid waste landfill. *CYP153* genes were found in samples of soils of all uses.

The results of PCA showed a significant effect of the PAH concentration in soils on the distribution of biotransformation genes (Fig. 6b). Two PC were identified, accounting for 68.5% of the total variance. PC1 represented 43.1% of the total variance and was strongly comprised with *nahAc* and *CYP153* genes (0.875 and 0.716, respectively) along with NAP (0.857), BIP (0.835), and BkF (0.738), and also demonstrated moderate positive loadings for *alkB* gene (0.637), FLU (0.661), PHE (0.650), ANT (0.576), PYR (0.550), BbF (0.664), BaP (0.569), DBA (0.648), and ΣPAHs (0.656). PC2 accounted for 25.4% of total variance showed strong positive loadings for *bphA* gene (0.885) along with FLT (0.822), BaA (0.765), and BghiP (0.747), and moderate loading for BaP (0.580) and ΣPAHs (0.504).

4. Discussion

High anthropogenic pressure was a decisive factor in the selection of soil sampling points for this research. The high content of hydrocarbons (especially PAHs, biphenyls) and their various derivatives was of particular interest. MSW landfills were selected in the first place as the most relevant to this requirement, since they constantly receive organic waste of natural origin (for example, food and wood, plant waste), partially transformed by industry natural organic matter (paper, etc.), as well as organic products of the chemical industry (such as plastic packaging, household chemicals, cosmetics, etc.).

An important problem of MSW landfills is the formation of pollutants such as PAHs (Hajizadeh et al., 2011; Rochman et al., 2013; Dai et al., 2014; Melnyk et al., 2015; Swati et al., 2017; Petrovic et al., 2018; Koshlaf et al., 2019). In addition to the constant burning of organic waste, pyrolysis processes also take place at the landfills, since the organics are mixed with the soil and oxygen supply is significantly limited. These processes contribute to the formation of PAHs. The formation of a large part of PAHs occurs during pyrolysis of polyvinyl chloride (PVC), which is abundant in municipal solid waste (Rochman et al., 2013; Zhou et al., 2016).

Zhou et al. (2015) showed that the formation of PAHs is most promoted by the pyrolysis of polystyrene and PVC. A lesser number of PAHs are formed from polyethylene terephthalate and lignin. The formation of 2 and 4-ring PAHs, with the dominance of 2-ring PAHs (40–70 wt % raw materials), was shown during the pyrolysis of 9 different solid wastes: xylan, cellulose, lignin, pectin, starch, polyethylene, polystyrene, PVC and polyethylene terephthalate. It was noted that most PAHs are formed during the pyrolysis of plastics, rather than biomass. Lignin produces the largest amount of PAH among biomass. Among the formed PAHs, NAP dominates, as well as 1-methylnaphthalene and 2-methhylnaphthalene are the predominant ones. PHE and FLT are formed among 3-ring PAHs in significant quantities. During the pyrolysis of polystyrene, polyethylene terephthalate and PVC, BaA and chrysene prevail.

Also, microorganisms associated with the human microbiome enter the landfill along with the waste. Together with these microorganisms, an influx of ARGs takes place. The introduced microorganisms interact with natural saprophytic microorganisms and solid waste polygons become potential reservoirs of ARGs (Threedeach et al., 2012; Song et al., 2016; Sun et al., 2016; Yu et al., 2016; Zhang et al., 2016) - "hot

spots" of exchange and the spread of ARG.

Soil samples were taken at existing landfills, recently closed and long-restored, i.e. located at different stages of the "life cycle" of MSW landfills. We also selected soils from the outskirts of two rural settlements, where the annual source of PAHs is the annual burning of dry grass.

In order to assess the impact of industrial chemical pollution, 12 soil samples were taken on the territory of the dry lake Atamanskoye, which was more than 30 years old, until the 90s discharge and storage of liquid industrial waste from the "Khimvolokno" association was carried out. As a result of the strongest anthropogenic press, the sludge collector lake turned into a source of environmental pollutants. According to Privalenko and Cherkashina (2012), the soil of the bottom of a dry lake and its shores is substantially contaminated with PAHs, organometallic compounds, and heavy metals. Thus, this object is a site model that has acquired a powerful industrial impact.

Based on the results of the studies outlined above, we had reasons to believe that the more PAH contaminated the soil is, the more different ARGs we can find in it. Previous studies by Sun et al. (2015a), Sun et al. (2015b) and Chen et al. (2017) do indeed support this concept. In addition, the work of our group shows that exposure to hydrocarbons causes oxidative stress in the bacterial cell and activates the SOS response. This can enhance horizontal gene transfer within the microbiome of hydrocarbon-contaminated soil.

Of all the soils studied in this work, the largest amount of PAHs was contained in technogenically contaminated soils. PAHs from these soils had pyrogenic nature. It is interesting that a wide range of ARGs (6 out of 10) was found in samples of technogenically contaminated soil that does not carry a recreational load and where there are no household or medical waste sources of ARB and ARG. And it was precise in technogenically contaminated samples that the maximum integral toxicity was detected (50% of the samples had a high degree of toxicity). Such a high level of technogenic load most likely caused the accumulation of a wide range of ARG, which appeared on the basis of natural microbiomes.

At the same time, as can be seen from the PCA results (Fig. 6a), we were unable to establish a reliable relationship between the ARGs content and the PAHs concentration. It is likely that the concentration of ARGs in the studied soils, in addition to the concentration of PAHs, is also affected by other factors that were not taken into account in this study.

On the other hand, PCA of hydrocarbon biotransformation genes showed a significant correlation with the presence of PAHs (Fig. 6b). A particularly close interrelation was shown by naphthalene dioxygenase (*nahAc*) and alkane monooxygenase *CYP153* (cytochrome of the P450 family). A significant correlation was also noted for the genes of biphenyl dioxygenase (*bphA*) and alkane hydroxylase (*alkB*). Moreover, *alkB* showed a correlation with the largest spectrum of PAHs. That is, contamination with PAHs stimulates the spread of genetic material associated with the degradation of alkanes rather than aromatic hydrocarbons (*CYP153* and *alkB*).

Assessment of integral toxicity using the *V. aquamarinus* VKPM B-11245 whole-cell bacterial lux-biosensor showed a high level of correlation both with the total content of PAHs and with the concentrations of individual PAHs (especially high-molecular PAHs, such as BghiP and BkF) in the samples of the studied soils. Similar results were obtained earlier for surface sediments. Given the high sensitivity, speed and low cost of the analysis, it is convenient to use this biosensor for preliminary ecotoxicological screening. Determination of PAHs content in soil samples is rational only at $T \geq 15$.

Evaluation of genotoxic effects using genetically engineered lux biosensors based on the *E. coli* MG1655 strain is significantly influenced by both the total content of PAHs and the ratio of PAHs with different numbers of rings in the samples. In clusters 1 and 2 with high and medium content of PAHs, with the prevalence of 5-ring (1 cluster) and 4-ring (2 cluster) compounds, a correlation of genotoxic effects with a small number of predominantly high-molecular PAHs was revealed.

With a decrease in the PAHs content in the samples, genotoxic effects begin to prevail over integral toxicity. Thus, in the samples taken in the least contaminated cluster 3, a close positive correlation ($r > 0.8$) was established between the genotoxic effects of the *E. coli* MG1655 biosensor (pColD-lux) without metabolic activation and the content of 2-, 3-, 4- and 5-ring PAHs (NAP, FLU, PHE, FLT, PYR, BaA, BghiP), as well as the total content of PAHs.

Thus, when interpreting the response of genetically engineered biosensors using promoters of various stress genes, it is necessary to take into account the qualitative and quantitative composition of pollutants in the sample. In addition, a panel of biosensors with promoters sensitive to various disorders of cellular metabolism is required to adequately assess the various biological effects of pollutants (Elad and Belkin, 2017).

5. Conclusions

As a result of studies 20 soil samples taken in the territory of MSW landfills, anthropogenic contaminated land and rural settlements in the southwestern part of the Rostov Region of Russia were studied.

A close correlation was established between the content of PAHs, genes for biotransformation of hydrocarbons (*nahAc*, *bphA*, *CYP153*, and *alkB*), and the integral soil toxicity, assessed using the whole-cell bacterial lux-biosensor *Vibrio aquamarinus* VKPM B-11245.

It was not possible to determine the correlation between the content of ARGs and PAHs within the framework of this study. However, in technogenically polluted soils richest in PAHs, the maximum diversity of the studied clinically relevant ARGs was found.

Since there are few publications confirming the relationship between PAHs and ARGs contamination and the fact that PAHs can enhance horizontal gene transfer and exhibit mutagenic effects, this issue remains poorly understood. The impact of various pollutants on the distribution and accumulation of soil ARGs in combination with environmental factors is an important and urgent topic for further research.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.110715.

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