

ADAPTATIVE RESPONSE OF FUNCTIONAL MICROBIOMES IN HEAVY METALS SOILS EXPOSED TO A PHYTOREMEDIATION PROCESS

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Abstract

The current context of land restoration and increasing the quality of urban ecosystems represent a challenge in the areas where historical pollution with heavy metals is present. The aim of this paper is to show the presence of adaptative mechanisms in the functional microbiome of 5 urban soils after a phytoremediation process. Total activities revealed that 3-7 times increase in the activity is visible in the first 72 hours of incubation and is almost double after 96 hours. This indicates an adapted microbiome to heavy metals conditions and its ability to develop as a community. An in-depth analysis reveals that carbohydrates and carboxylic acids were the most prominent guilds from the community, but with a distinct functional signature in each of the analyzed sites. Interestingly, polymers, amino acids and amines/amides guilds which represent almost 40% of the activity reported to the entire community, even though these guilds have a reduced functional profile. The results indicate that plant presence in polluted ecosystems represents a factor that enables the recovery of functional microbiome and maintenance of a high activity.

Key words: functional proliferation, heavy metal tolerance, site-specific activity, functional patterns, community assemblage.

INTRODUCTION

The changes in environmental conditions can modify the structure and functioning of soil microbial communities (Raj et al., 2017). In this context, maintaining ecosystem health requires the conservation of biodiversity and the continuous monitoring of changes at all trophic levels, including the microbial functions (Ojuederie et al., 2017). Soil microbes play a key role in element cycles, organic matter decomposition, energy flow, and soil structure maintenance (Ju et al., 2016; Borowik et al., 2018; Lombard et al., 2011).

In the category of environmental pollutants, heavy metals are known to disturb soil biological activity. These metals cause both biological and chemical soil degradation (Masindi et al., 2018), negatively impacting microbial populations and enzyme activity. They affect both microbial diversity, community functions, assemblage and soil biochemical processes (He et al., 2010; Kavamura et al., 2010).

The distribution of heavy metals in the environment is determined by environmental conditions and the specific characteristics of

metals (Khlifi et al., 2010). This type of pollution negatively affects plant growth and development. However, plants have developed various adaptation mechanisms, especially due to their rhizosphere microbiome (Mishra et al., 2017; Agarwal et al., 2024), that allow them to survive under stressful conditions in contaminated environments. Metallophytes are plants capable of tolerating high concentrations of heavy metals, have adapted to mineral substrates, including those rich in metals (Alford et al., 2010; Khan et al., 2009). In contrast, pseudometallophytes are plant species that can grow on both uncontaminated soils and soils polluted with heavy metals (Becerra-Castro et al., 2012).

Due to its safe and environmentally friendly nature, phytoremediation is an increasingly preferred method for cleaning up the environment. Genetic improvement of hyperaccumulator plants has led to an increase in their ability to retain metals, and sustainable approaches allow pollutants to be removed without compromising agricultural activities (Sharma et al., 2025). The accumulation of heavy metals in plants affects cellular processes and development, diminishing the effectiveness

of phytoremediation; therefore, assessing their impact is essential (Rasheed et al., 2020; Raza et al., 2021).

The aim of this research was to assess the functional assemblage of microbial communities from 5 sites supposed to three years of phytoremediation. The objectives were: i) to analyze the assemblage of functional microbiome during a period of 96 hours of incubation; ii) to assess the activity correlation trends within functional groups in 24 hours periods; iii) to explore the general direction of guild activities toward sites conditions.

MATERIALS AND METHODS

The methodological information presented here has been adapted and developed based on a previously published study by Pop et al., 2025. The daily dynamics of the functional soil microbiome were assessed using the Biolog EcoPlate system at five locations in the municipality of Baia Mare. The sites were chosen due to their historical pollution and were supposed to 3 years phytoremediation process. The investigated areas are identified by the following abbreviations: CR (Craica), CT (Colonia Topitorilor), FR (Fernezuiu), ROMP (Romplumb), and URB (Urbis). Soil samples were collected in 2023 from the long-term experiment initiated in 2019 (47°39' N, 23°34' E) in North-Western Romania, covering a total area of approximately 7.3 hectares of degraded industrial land. EcoPlate analyses were performed according to the methodology described by Weber et al. (2009), with substrates grouped according to chemical similarity. The soil suspension was diluted to 10^{-4} , and absorption was measured at 590 nm using a microplate reader. Monitoring was conducted over a period of five days until the plateau phase was reached, characterized by the stabilization of absorption values.

The Biolog EcoPlate™ method provides no details about the genetic composition of microbial communities, but highlights how bacteria use substrates and their metabolic potential in stressful environments, such as contaminated ones. Its simplicity, low cost, speed, and reproducibility make this method suitable for environmental monitoring as well. (Nemeth et al., 2021).

Data analysis was performed in the RStudio environment, and the flow was based on a general to specific range of tests (R Core Team, 2024). Non-Metric Multidimensional Scaling (NMDS) showed the differences between communities at different incubations times (Oksanen et al., 2022). These projections enabled the correlation between community stability, dispersion of data and the orientation of guild vectors.

At each of the 48, 72 and 96 hours of readings were extracted the Pearson correlations between functional groups and the most significant ones were used as a selection tool for the further analysis at site level. Each group that showed significant positive or negative correlation was analysed with ANOVA and LSD test for the assessment of both importance within community and the presence of differences between sites (de Mendiburu., 2023). The extraction of most correlated groups was performed due to their activity within the entire community and the number of links with other groups. This process enables both the analysis of the most important groups within the community and the real differences between the activity performed in one site.

RESULTS AND DISCUSSIONS

NMDS analysis (Figure 1, Table 1) shows how microbial communities at the five sites (CR, CT, FR, ROMP, and URB) are grouped according to how they use carbon sources, highlighting the influence of historical pollution in Baia Mare on soil metabolic activity. The most important factors explaining the differences between sites are AWCD and SUM, indicators of general metabolic activity. Their orientation along the NMDS1 axis indicates that the samples located on the left side of the graph, especially those from FR (Fernezuiu), show more intense metabolic activity and more efficient use of substrates compared to the other sites.

Carbohydrates (CH) and carboxylic (CX) acids are the main sources of energy for microorganisms, especially under conditions of chemical stress. In contrast, the use of amino acids (AA), amines (AM), and polymers is lower and occurs mainly in sites more affected by metal pollution, such as URB and CT. This pattern shows that although heavy metals affect

soil quality, microbial communities have adapted, maintaining certain metabolic functions essential for the circulation of energy and elements.

At 48 hours, most functional guilds show a significant correlation with the whole microbiome structure. The most significant relationships are observed for SUM, as for the AA, CH, and CX guilds, which present highly significant activity. AWCD indicates significant metabolic activity, suggesting a general intensification of substrate utilization. In contrast, the P guild does not show a statistically significant value which indicates a low level of contribution to the functional diversity of the microbiome.

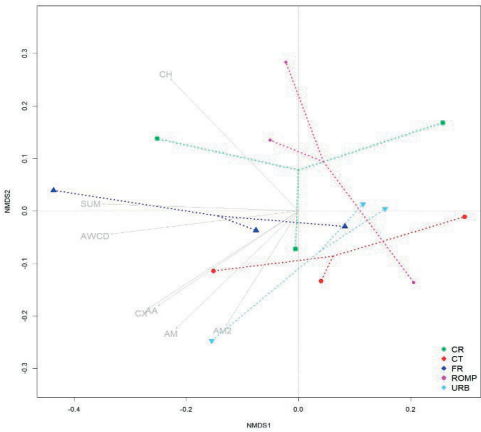


Figure 1. NMDS projection of functional microbiomes in the 5 targeted sites at 48 h

Table 1. Functional guild and groups traits in relation to the entire microbiome (48 h)

	NMDS1	NMDS2	r2	p.val	
AA	-0.9984	0.0566	0.8554	0.001	***
AM	-0.97809	0.20818	0.7059	0.002	**
AWCD	-0.9835	0.18092	0.5598	0.011	*
CH	-0.47266	-0.88124	0.787	0.001	***
CX	-0.97916	0.20308	0.8543	0.001	***
P	-0.72146	-0.69246	0.5008	0.072	.
SUM	-0.93325	-0.35924	0.9769	0.001	***

Note: ns - not significant activity; * - significant activity; ** - distinct significant activity; *** - very significant activity.

At this point of incubation, there is a visible large angle between vectors, with CH positioned toward the -/+ quadrant where points from FR and CR are visible, and CX, AA and AM vectors

that are positioned in the -/- quadrant. The last vectors indicate a strong association in the incipient community, with coupled activities and positive interactions. One interesting case is the CX and AA orientation toward the same direction, indicating a strong positive correlation and a synergic effect in the transformation of the community assemblage. The position of Sum vector is in the middle distance between the marginal vectors, suggesting that each of the guild activities could produce a significant shift toward CH or CX, AA and AM guilds.

The interpretation of the NMDS analysis (Figure 2, Table 2) highlights a clearly differentiated pattern among the sites in Baia Mare based on the metabolic capacity of microbial communities, emphasizing the major role of historical pollution and local environmental conditions. The sites located on the left side of the graphic, in the AWCD and SUM areas, especially the FR site and partially the URB site, show more intense biological activity and more efficient use of substrates, suggesting a functional adaptation of the microbiome to heavy metal-induced stress.

At the same time, carbohydrates and polymers are closely correlated and have a major influence on the functional structure of the communities at the CR site, indicating a metabolism oriented to the degradation of complex organic compounds. For the URB site, carboxylic acids and amines/amides play an important role, reflecting a more diverse metabolic profile, while the ROMP and CT sites are situated on the right side of the graphic, opposite the metabolic activity vectors, indicating an inhibition of enzymatic processes and reduced functional diversity. This distribution confirms that, although the soil microbiome has the ability to adapt, high levels of pollution at industrial sites significantly limit the metabolic and functional potential of microbial communities.

After 72 hours, the metabolic activity of the microbial community is more pronounced, with several variables that are highly significant. The SUM, AWCD, CH, and CX indicators show high r² coefficient values and significant levels, which suggests an intense use of the substrates and a metabolic diversification of the microbiome. The AA and AM guilds also

remain significant, which confirms their importance in the functional community structure. As in the previous table, P remains statistically insignificant, indicating a reduced influence on microbiome variation. Both the sum and AWCD vectors are positioned inside the angle formed by CH and CX vectors, indicating a similar impact of microbiome shift of both guilds inside communities. The dispersion of points is moderate, sustaining a future larger dispersion associated with the potential diversification of metabolism within communities, even at site level. Polymer guild vector is positioned outside CH, indicating both the association between those two guilds and a potential increase of this guild in the community.

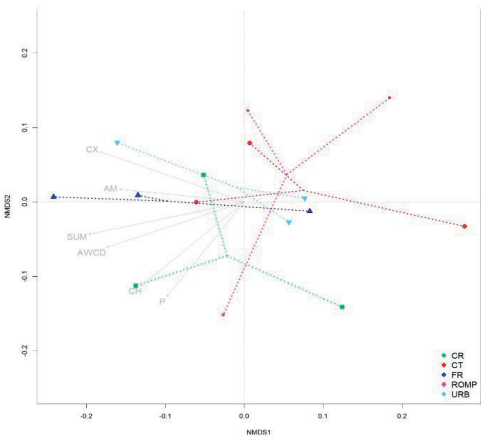


Figure 2. NMDS projection of functional microbiomes in the 5 targeted sites at 72 h

Table 2. Functional guild and groups traits in relation to the entire microbiome (72 h)

	NMDS1	NMDS2	r2	p.val	
AA	-0.81093	-0.58515	0.7284	0.001	***
AM	-0.69876	-0.71536	0.7537	0.002	**
AWCD	-0.99157	-0.12957	0.8725	0.001	***
CH	-0.67167	0.74085	0.8902	0.001	***
CX	-0.82171	-0.5699	0.8125	0.001	***
P	-0.93708	0.34912	0.4039	0.065	.
SUM	-0.99929	0.03768	0.9418	0.001	***

Note: ns – not significant activity; * - significant activity; ** - distinct significant activity; *** - very significant activity.

NMDS analysis (Figure 3, Table 3) based on microbial guilds shows that the distribution of sites in Baia Mare is closely related to the

metabolic capacity of bacterial communities exposed to heavy metal pollution. The FR and, partially, URB sites show the highest biological activity and functional diversity. Carbohydrates and carboxylic acids are the main functional groups that support metabolic activity in all analyzed sites. Guilds associated with amino acids and amines/amides are more strongly represented in sites with relatively stable functional activity, such as URB and CT. In contrast, the ROMP and CT sites are positioned opposite the metabolic activity vectors (SUM/AWCD), suggesting reduced enzymatic activity and limited functional potential in areas significantly affected by heavy metal pollution.

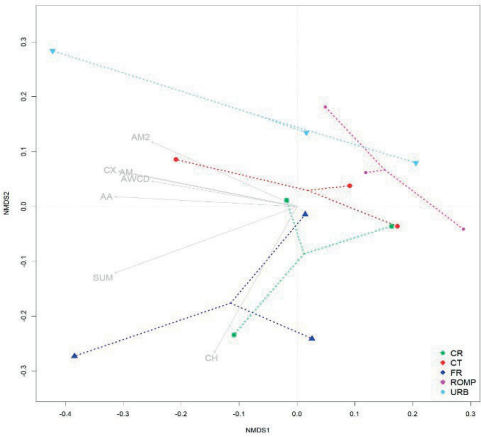


Figure 3. NMDS projection of functional microbiomes in the 5 targeted sites at 96 h

Table 3. Functional guild and groups traits in relation to the entire microbiome (96 h)

	NMDS1	NMDS2	r2	p.val	
AA	-0.9902	0.13965	0.2841	0.142	
AM	-0.99408	0.10864	0.5734	0.003	**
AWCD	-0.94402	-0.32988	0.7707	0.001	***
CH	-0.75725	-0.65312	0.6722	0.003	**
CX	-0.93796	0.34675	0.8508	0.001	***
P	-0.61248	-0.79049	0.5824	0.004	**
SUM	-0.97657	-0.21522	0.9184	0.001	***

Note: ns – not significant activity; * - significant activity; ** - distinct significant activity; *** - very significant activity.

At 96 hours, the highest metabolic activity is observed, with most variables showing significant or very significant correlations. SUM, AWCD, and CX have the highest r² values and very significant levels, which reflects

increased substrate use by the microorganism community. AM, CH, and P also show significant activity. The only insignificant variable is AA, which indicates that this functional group has a lower influence on microbiome diversity.

The entire sum of activities vector is oriented toward FR site, between CH and AA guild vectors, indicating that a change in these guilds activity will modify the entire activity of the microbiome. Most of the sites present dispersed communities, indicating a point-specific activity of the microbiome and a diversity of metabolic potentials within communities.

The values presented indicate how microbial communities at the five sites (CR, CT, FR, ROMP, and URB) utilize different carbon sources from the categories carbohydrates (CH), carboxylic acids (CX), polymers (P), and amino acids (AA) (Table 4). The FR (Ferneziiu) site has the highest values for most of the substrates analysed (CH1, CH3, CH6, CX4, AA5, and AA6), which suggests more intense metabolic activity and a higher capacity of the microbiome to use diverse carbon sources.

Table 4. Site-specific activity at 48 h based on group importance within community

Gro up	+CH1	+CH3	+CH6	+CX4	+P4	+AA 5	+AA6
CR	0.16± 0.02a	0.08± 0a	0.08± 0a	0.07± 0.01a	0.12± 0a	0.08± 0ab	0.08± 0ab
CT	0.15± 0.01a	0.07± 0a	0.07± 0a	0.06± 0a	0.11± 0a	0.07± 0ab	0.08± 0ab
FR	0.2±0. 04a	0.11± 0.03a	0.11± 0.03a	0.09± 0.02a	0.14± 0.03a	0.1±0 .02a	0.11± 0.02a
RO	0.13± 0.01a	0.06± 0a	0.06± 0a	0.05± 0a	0.09± 0a	0.07± 0b	0.07± 0b
MP	0.15± 0.04a	0.06± 0a	0.06± 0a	0.05± 0a	0.07± 0b	0.06± 0b	0.06± 0b
UR							
B							

Note: different letters indicate significant differences at $p < 0.05$ based on LSD test. Groups preceded by + indicate positive interactions.

In contrast, the ROMP and URB sites show lower values, especially for amino acids, which indicates a decrease in microbial activity under stress caused by heavy metals. For carbohydrates and carboxylic acids, the differences between sites are not as pronounced, meaning these substrates are still the main energy sources for microorganisms even when there's contamination. The results show that the metabolic activity of the microbiome is better sustained in the FR site, while in sites more severely affected by pollution, is observed a decline in the use of certain substrates.

Table 5 includes both positive (+) and negative (−) guilds, reflecting substrates that are

positively or negatively associated with microbial metabolic activity. The results indicate that the FR site has the highest values for most substrates in the positive guilds, particularly +CH1, +CH7, and +AA1, which confirms higher metabolic activity and greater functional potential of the microbial communities in this area.

In contrast, the ROMP and URB sites show lower values for some positive guilds, particularly +AA1 and +P4, which may indicate a reduced capacity of microorganisms to utilize specific substrates under severe heavy metal contamination conditions. The lower values observed for +P4 at all sites suggest that the degradation of polymeric substrates is generally limited, most likely due to the higher enzymatic requirements necessary for the decomposition of complex compounds.

Regarding the negative guilds (−AA4 and −CX7), the relatively higher values at some sites may indicate the presence of metabolic limitations or changes in substrate preference under environmental stress conditions. These trends highlight clear functional differences between microbial communities and suggest that the FR site has a higher adaptive capacity, maintaining higher metabolic activity even under conditions of historical contamination

Table 5. Site-specific activity at 72 h based on group importance within the community

Grou p	+CH1	+CH7	+P4	+AA1	−AA4	−CX7
CR	0.16±0.0 2a	0.08±0a	0.12±0a	0.08±0a b	0.75±0.0 1a	0.50±0.13 ab
CT	0.15±0.0 1a	0.07±0a b	0.11±0a b	0.07±0a b	0.70±0.0 6a	0.65±0.29 ab
FR	0.2±0.04 a	0.11±0.0 3a	0.14±0.0 3a	0.1±0.0 2a	0.90±0.2 0a	0.39±0.24 a
ROM	0.13±0.0 1a	0.06±0a	0.09±0a	0.07±0b	0.62±0.1 0a	0.52±0.20 b
P	0.15±0.0 4a	0.06±0a	0.07±0b	0.06±0b	0.62±0.0 2a	0.58±0.14 b
URB						

Note: different letters indicate significant differences at $p < 0.05$ based on LSD test. Groups preceded by + indicate positive interactions; groups preceded by − indicate negative interactions.

Table 6 shows the use of several substrates of the carbohydrate (CH7, CH10), carboxylic acid (CX3, CX4), and amine (AM1) groups by microbial communities. The results show that the FR site has the highest values for most substrates, such as CH7 and CX3, which indicates high metabolic activity and more functional diversity of the soil microbiome at this location.

In contrast, the ROMP site shows lower values for certain substrates, particularly CX3,

suggesting a decrease in microbial metabolic activity that may be associated with a higher level of heavy metal contamination. The lower utilization of carboxylic acids may reflect disruptions in microbial metabolic processes involved in the carbon cycle.

The use of amines (AM1) is more pronounced at the FR and URB sites, which may indicate the presence of microbial groups able to use alternative nitrogen-containing substrates as an adaptive response to environmental stress conditions (Krysenko et al., 2025).

The results suggest that despite heavy metal pollution influencing the metabolic functioning of the soil microbiome, microbial communities maintain to some degree their ability to utilize different carbon sources (Ma et al., 2022). However, the intensity of substrate utilization varies between sites, reflecting different levels of microbe community recovery and adaptation after remediation (Yin et al., 2024).

Table 6. Site-specific activity at 96 h based on group importance within the community

Site	+CH7	+CH10	+CX3	+CX4	+AM1
CR	1.34±0.24a	0.24±0.02a	1.67±0.15ab	0.36±0.15ab	0.34±0.13a
CT	0.99±0.07a	0.18±0.05a	1.90±0.20ab	0.22±0.07b	0.52±0.35a
FR	1.72±0.46a	0.39±0.12a	2.15±0.15a	0.74±0.19a	0.91±0.42a
ROMP	1.16±0.14a	0.30±0.03a	1.43±0.08b	0.25±0.02ab	0.36±0.07a
URB	1.04±0.11a	0.23±0.02a	1.88±0.21ab	0.39±0.17ab	0.72±0.38a
Site	-CH3	-CH9	-CX9	-AA4	-AA5
CR	0.81±0.28a	1.47±0.33a	0.91±0.51a	1.35±0.13a	0.82±0.36a
CT	0.46±0.10a	0.72±0.32b	1.02±0.41a	1.66±0.38a	1.08±0.28a
FR	0.62±0.13a	1.11±0.08ab	1.32±0.43a	1.56±0.33a	0.95±0.09a
ROMP	0.58±0.01a	1.16±0.18ab	1.35±0.60a	1.06±0.05a	0.92±0.27a
URB	0.42±0.16a	1.03±0.17ab	0.81±0.42a	1.54±0.13a	0.63±0.15a

Note: different letters indicate significant differences at $p < 0.05$ based on LSD test. Groups preceded by + indicate positive interactions; groups preceded by – indicate negative interactions.

Phytoremediation is an important example of bioremediation, based on the use of plants to accumulate, remove, or reduce the level of pollutants in the soil (Ali et al., 2013). The method is considered effective due to its direct application on site, the preservation of the biological and structural characteristics of the soil, and its significantly reduced costs, which are up to 100 times lower than those of physical-chemical methods (Stefani et al., 2015).

Improving phytoremediation is essential for limiting the bioavailability and transport of heavy metals to the aerial parts of plants, reducing the risk of biomagnification in the food chain. Plants adapted to heavy metal-contaminated soils exhibit specific survival

strategies and traits-such as tolerance to unfavorable soil conditions, extensive root systems, and high biomass production-that make them suitable for use in phytoremediation (Yang et al., 2005).

These locations have different levels of heavy metal contamination, mainly generated by mining and metallurgical activities, as well as urban development (Pop et al., 2026). The study results highlight that the phytoremediation applied to soils contaminated with heavy metals in Baia Mare affected the functional structure and metabolic activity of soil microbial communities. Analyses conducted with Biolog EcoPlate indicate differences between the sites investigated in the capacity of microorganisms to utilize different carbon sources, which reflects varying levels of adaptation to heavy metal stress.

The Ferneziu site (FR) shows the highest metabolic activity values and the most efficient carbon substrate utilization capacity, which suggests a more advanced functional restoration of the microbiome after the remediation process. This shows that, under specific conditions, microbial communities can develop effective mechanisms of adaptation and maintain active metabolic activity in soils that have historically suffered from pollution.

In contrast, sites such as Romplumb (ROMP) and, Colonia Topitorilor (CT) show lower values for the utilization of specific metabolic substrates, especially amino acids and carboxylic acids, which indicates that these sites are still affected by contamination and that microbiological remediation is less advanced. Limited use of more complex substrates may indicate restrictions in the enzymatic activity and functional diversity of microbial communities (Kandeler et al., 1996).

The results confirm that, in the post-remediation phase, the soil microbiome can maintain important metabolic functions, especially the use of carbohydrates and other simple carbon sources, which are the major energy substrates for microorganisms under environmental stress. Overall, the study highlights that phytoremediation contributes to the gradual restoration of soil microbiome function, but the level of recovery is different from site to site, based on the initial level of contamination and local environmental conditions (Sekhohola-

Dlamini et al., 2022). Functional microbial community analysis is a useful tool in the evaluation of the effectiveness of remediation processes for heavy metal-contaminated soils. Colonia Topitorilor (CT) sites recorded lower values for the use of some substrates, especially amino acids and carboxylic acids, suggesting a slower microbiological remediation.

CONCLUSIONS

Phytoremediation used in soils contaminated with heavy metals in Baia Mare impacted the functional structure and metabolic activity of microbial communities. Biolog EcoPlate analyses highlighted differences between sites in terms of carbon source utilization, which indicated different levels of adaptation to heavy metal stress. The Ferneziu (FR) site showed the highest metabolic activity and the most efficient use of substrates, suggesting a more advanced functional restoration of the soil microbiome. In contrast, the Romplumb (ROMP) and Colonia Topitorilor (CT) sites recorded lower values for the use of some substrates, especially amino acids and carboxylic acids, suggesting a slower microbiological remediation.

ACKNOWLEDGEMENTS

This paper is part of a PhD study in the thematic area of “*The traceability of heavy metals in historically polluted sites and the possibilities for their return to agriculture*”, conducted by the first author B.P., under the coordination of Prof. dr. Roxana Vidican (R.V.).

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