

IMPACT OF HEAVY METAL POLLUTION ON SOIL BACTERIAL CARBON METABOLISM

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Abstract

The aim of the study was to evaluate the long-term impact of heavy metals on soil bacterial metabolism by assessing their capacity to utilize a set of carbon sources. The study area is in the vicinity of the Pb/Zn smelter KCM 2000, South-Central Bulgaria, with considerable Pb, Zn, Cd, Cu, and As pollution. The Biolog Ecoplate approach was used to evaluate the bacterial metabolic activity, expressed by the average well colour development; the bacterial metabolic fingerprints expressed by community-level physiological profiling, and Shannon bacterial functional diversity. The results revealed reduced metabolic activity recorded for bacterial communities inhabiting the heavily polluted soils. The most preferred carbon groups utilized by the bacterial communities were the polymers, followed by carbohydrates and amino acids. Local variability of soil properties, such as inorganic ions, total organic carbon, pH, soil moisture, and soil texture, could modify the effects of heavy metals. This study highlighted that Biolog Ecoplate™ is a suitable approach for assessing bacterial functional shifts in relation to long-term soil pollution stress.

Key words: heavy metal pollution, soil bacterial community, metabolic profiles, AWCD, CLPP

Introduction. Soil microbial communities play a crucial role in almost all biogeochemical processes in terrestrial ecosystems, including organic matter decomposition, nutrient cycling, and plant productivity [1]. Smelting activities pose

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serious environmental challenges due to the accumulation of heavy metals (HMs) in surrounding soils, leading to local pollution and serious ecological risk for soil inhabitants [2].

Numerous studies have shown that HM pollution significantly impacts the diversity, structure, and function of microbial communities [3–5]. Nevertheless, some studies have reported that HM pollution exerts only a limited influence on microbial community structure, suggesting that abiotic factors may also play a significant role in shaping microbial activity [6]. Most of the recent studies focus on the taxonomic and predictive functional profiling of microbial communities based on metagenomic data obtained through high-throughput sequencing (Next-Generation Sequencing – NGS) technologies [7]. Despite this, NGS platforms still have limitations in providing insights into the metabolic potential of microbial communities, which is critical for describing their practical significance for the implementation of ecological processes in the ecosystem [5]. To overcome these limitations, a community-level physiological profiling (CLPP) approach using Biolog Ecoplate™ test was used. This test provides a rapid assessment of bacterial metabolic capacity by measuring the utilization of diverse carbon sources [5, 8, 9]. The method helps to evaluate the ecological status of soils affected by HM pollution.

This study aims to evaluate the long-term impact of HMs on soil bacterial metabolism by assessing their capacity to utilize a set of carbon sources.

The study area is in the vicinity of the Pb/Zn smelter KCM 2000, situated in the Plovdiv region of South-Central Bulgaria. Its long-term smelting activity significantly affected the local environment, resulting in considerable soil pollution with Pb, Zn, Cd, Cu, and As.

Materials and methods. Soil sampling. Five sampling sites with increasing distance from the source of pollution were selected: KCM_1 – 0.5 km south of the smelter (42°03'31.68"N, 24°49'19.2"E); KCM_2 – 2 km south of the smelter (42°03'5.76"N, 24°49'19.6"E); KCM_3 – 3 km south of the smelter (42°02'6"N, 24°49'19.2"E); KCM_4 – 1 km southeast of the smelter (42°03'31.68"N, 24°49'45.12"E); KCM_5 – 1 km northeast of the smelter (42°04'23.52"N, 24°49'45.12"E). At each site, three soil samples were collected from two depths: surface (0–20 cm) and sub-surface (20–40 cm), and then combined into aliquots to create representative samples for each site and depth. The representative samples were analyzed for HM concentrations and bacterial metabolic activities.

Soil physicochemical properties and heavy metal/metalloid concentrations. The concentrations of Zn, Pb, Cd, Cu, As, and the soil physicochemical properties were measured according to [10].

Catabolic profiles of the bacterial community. The Biolog Ecoplates™ system (Biolog Inc., Hayward, CA, USA), containing 31 different carbon sources (CSs) in three replicates, was used to evaluate community-level physiological profiles (CLPPs) under HM stress. The wells of the microplates were inoculated

with 120 μL of bacterial cell suspensions (1 g soil in 50 ml sterile 0.9% NaCl, suspended for 30 min at 220 rpm, followed by filtration through 8.0 μm and 3.0 μm pore size membranes) and incubated at 25 $^{\circ}\text{C}$ in the dark, as described in [11]. Substrate utilization was monitored by measuring absorbance at 590 nm over 5 days using a Microplate Reader LKB 5060-006. Biolog CSs were grouped into five chemical groups (CGs) based on their chemical moieties: carbohydrates (CH), polymers (POL), carboxylic acids (CA), amino acids (AA), and amines/amides (AM) [11]. The Biolog-derived data were used to evaluate: (1) bacterial metabolic activity, expressed by the average well colour development (AWCD); (2) bacterial metabolic profiling expressed by CLPP, and (3) bacterial functional diversity (Shannon, H').

Data analysis. The microbial data presented in this paper are expressed as means $\pm$ standard deviation (SD). To assess differences in bacterial metabolic activity between sites, an ANOVA was conducted, followed by post-hoc Tukey's tests. Canonical correspondence analysis (CCA), was performed to evaluate the similarity among soil bacterial communities and estimate the relationships between bacterial metabolic profiles and soil properties. Data processing and visualization were performed using the software PAST 4.13 at $p < 0.05$.

Results and discussion. Soil physicochemical properties and heavy metal/metalloid concentrations. The concentrations of HMs exceeded several times the soil maximum permissible concentrations specified by Bulgarian Regulation 3/2008 (<https://www.moew.government.bg/bg/pochvi/zakonodatelstvo/nacionalno-zakonodatelstvo/>) (Table 1). According to our previous results, the levels of soil pollution were classified as: heavy (KCM_1, KCM_2 and KCM_4), moderate (KCM_5), and slight (KCM_3) [10].

The soil texture was categorized as sandy loam. The soil pH varied from slightly acidic (pH 6.7) to neutral (pH 7.2). The soil moisture (SM) differed between the soils: from 6.7% (KCM_3.2) to 22.7% (KCM_5.1). The total organic carbon (TOC) varied from 7.04 g/kg (KCM_5.1) to 16.07 g/kg (KCM_4.2). The inorganic ammonium ($\text{NH}_4\text{-N}$), and nitrate ($\text{NO}_3\text{-N}$) nitrogen, and phosphate (PO_4) ranged from 2.22 mg/g (KCM_5.1) to 6.84 mg/g (KCM_1.2), from 3.01 mg/g (KCM_3.1) to 43.38 mg/g (KCM_1.1), and from 4.18 mg/g (KCM_3.2) to 29.8 mg/g (KCM_5.1), respectively.

Community level metabolic activity (AWCD). The average metabolic activity (AWCD) served as an indicator of the capacity of soil bacterial communities to utilize a set of 31 different carbon sources. AWCD values varied across the sampling sites, and the metabolic activities are presented in Fig. 1. The metabolic capacity of soil bacterial communities ranged from 0.178 ± 0.042 OD (KCM_5.1) to 0.615 ± 0.081 OD (KCM_3.2).

Bacterial communities from KCM_1.1 (0.186 ± 0.017 OD), KCM_1.2 (0.182 ± 0.067 OD), and KCM_5.1 (0.178 ± 0.042 OD) exhibited low metabolic capacity. A higher metabolic potential was recorded for bacterial communities inhabiting

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Soil concentrations of heavy metals/metalloid in the area of
KCM 2000

Soils	Concentration of heavy metals (mg/kg)				
	Zn	Pb	Cd	Cu	As
KCM_1.1	9452	11569	184.9	891.4	191
KCM_2.1	1558.2	1370.1	15.7	138.9	13.4
KCM_3.1	216.2	135.6	3.9	53.7	9.6
KCM_4.1	6872	5723	86.2	570.3	80
KCM_5.1	740	335	9.3	98.2	15.5
KCM_1.2	3457.1	3744	61.8	331.1	60.6
KCM_2.2	1711.9	1480.3	38.6	155.1	5
KCM_3.2	250	130	3.6	60	9
KCM_4.2	4843.1	3424	55.7	341.5	63.5
KCM_5.2	810	330	9	88.2	12.7
MPC*	320	100	2	150	25

MPC* – maximum permissible concentrations of HMs (mg/kg) according to Regulation 3/2008 in permanent grass-land soils at < 6.00 pH; Heavy metal concentrations that exceeded Bulgarian MPC are given in bold.

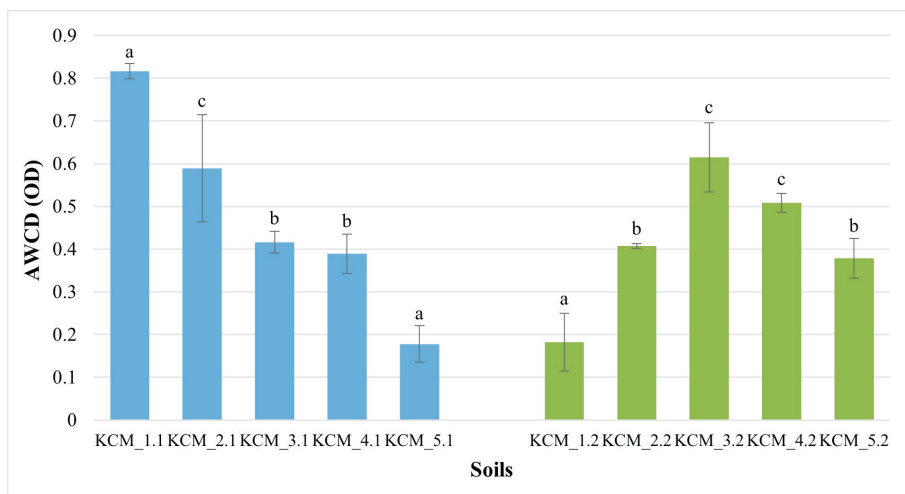


Fig. 1. Capacity (AWCD) to utilize Biolog carbon sources of bacterial communities from surface (KCM_1.1 – KCM_5.1) and subsurface (KCM_1.2 – KCM_5.2) soil layers in the area of KCM 2000. Different lowercase letters indicate a statistically significant difference ($p < 0.05$) between AWCDs

the slightly polluted soil of KCM_3.2 (0.615 ± 0.081 OD), as well as that of KCM_2.1 (0.589 ± 0.125 OD) and KCM_4.2 (0.508 ± 0.022 OD), both of which

had higher levels of TOC (14.07 g/kg and 16.7 g/kg, respectively). The reduced metabolic activity in heavily polluted soils can be attributed to decreased abundances of heterotrophic bacteria, altered primarily by the high HM concentrations or/and relatively unfavourable local environmental conditions, reported also as a main factor in [5]. These findings align with previous studies [4, 11, 12], which also reported a decline in bacterial capacity to catabolize carbon sources under metal stress.

Metabolic profiling of bacterial community (CLPP). Soil bacteria exhibit distinct preferences and varying capacities for utilizing Biolog CSs. Because Biolog substrates differ in their chemical structure, they are classified into five groups (CGs) based on their chemical composition, and the capacity of soil bacteria to utilize each of these CGs was evaluated. The contribution of each CG utilization rate to the total bacterial activity was shown in Fig. 2.

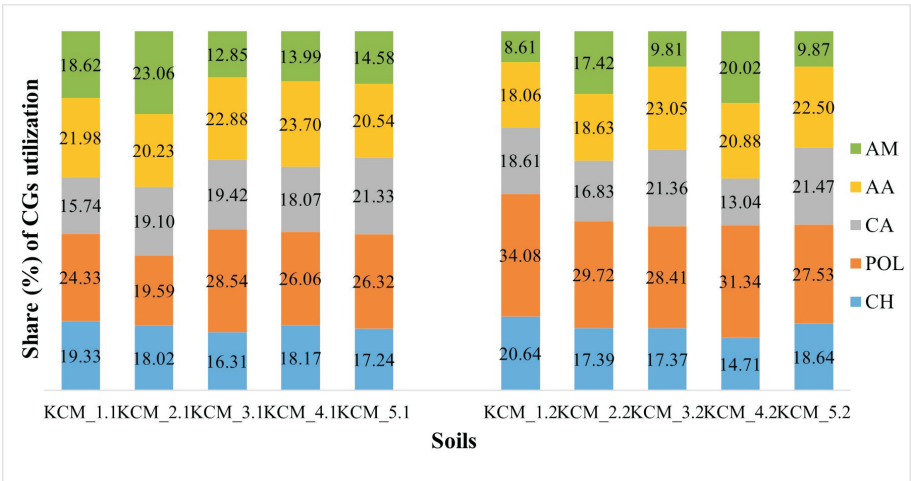


Fig. 2. Capacity of soil bacterial communities to utilize different carbon groups (CG), expressed as a percentage of total metabolic activity (AA – amino acids, CA – carboxylic acids, CH – carbohydrates, POL – polymers, AM – amines)

The results demonstrated relatively uniform metabolism of the different CGs across all soils. The utilization of CH accounted for $17 \pm 1.6\%$ of total bacterial activity, POL for $27.6 \pm 4\%$, CA for $18.5 \pm 2.7\%$, AA for $21.3 \pm 1.9\%$, and AM for $14.9 \pm 4.8\%$. The metabolic profiles of CG utilization showed that POL were most preferred by the bacterial communities, followed by CH and AA (Fig. 2). This preference aligns with bacterial adaptive strategies, which tend to exploit the most abundant carbon sources in their environment, typically polymers, whose breakdown provides substantial energy [8]. Additionally, it is plausible that HM pollution increases the cellular energy demand needed for maintaining detoxification pathways, thereby promoting the preferential degradation of CHs, which yield the highest energy per utilizable molecule. AA, in turn, serves as a

source of both carbon and nitrogen. Recent findings [13] also indicate that microbial communities under stress prefer to utilize AA. Similar patterns of increased utilization of POL, CH, and AA in HM-polluted soils have been documented by other researchers [9, 11].

The CLPPs data were used to assess the functional diversity of bacterial communities from both soil layers. The Shannon index ranged from 3.40 ± 0.17 in KCM_2.1 to 3.54 ± 0.12 in KCM_1.1 in the surface layer, and from 3.40 ± 0.12 in KCM_3.2 to 3.46 ± 0.36 in KCM_5.2 in the subsurface layer. Although the observed variation was minimal and statistically insignificant ($p > 0.05$), the consistently high values indicate substantial functional diversity across sites and soil layers. These findings suggest that broad-spectrum metabolic activity remains well preserved despite elevated HM pollution. Such resilience is consistent with the prolonged exposure of these soils to pollutants and reflects the capacity of microbial communities to adapt to persistent environmental stress.

Relationship between bacterial metabolic profiles and soil properties. To determine which abiotic factors have modulatory effects on metabolic bacterial profiles, a canonical correspondence analysis (CCA) was performed (Fig. 3).

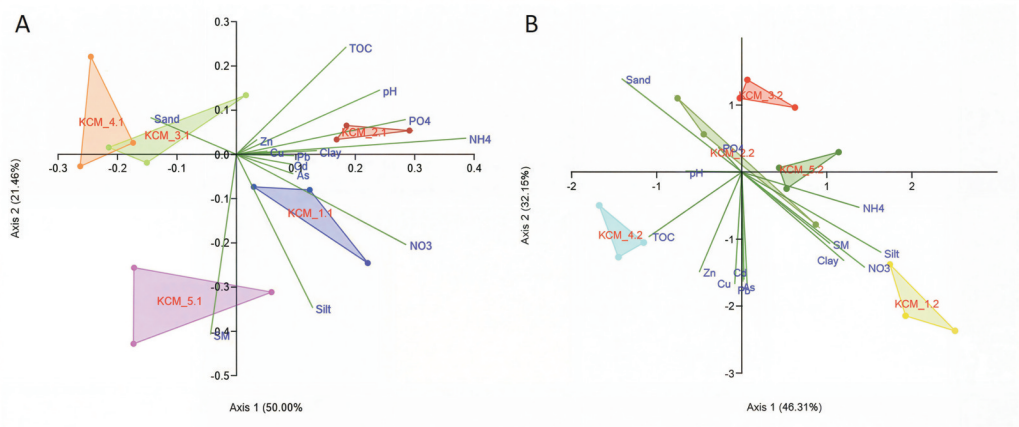


Fig. 3. Canonical correspondence analysis (CCA) of bacterial metabolic profiles (CLPPs) and soil properties of (A) surface- and (B) subsurface layers in the area of KCM 2000

The first two canonical axes were used for ordination, which contain the maximum expressed variation of 71.46% (Fig. 3A) and 78.46% (Fig. 3B). According to the CCA results, HM concentrations have a low influence on bacterial metabolic profiling, except for their negative effects on bacteria from subsurface soil at KCM_3.2. The results show that soil properties, such as inorganic ions, TOC, pH, SM, and soil texture, had a much stronger influence on bacterial metabolic profiles than HMs. The influence of soil properties on bacterial functions differed between soil depths. For example, pH and nutrient levels played a major role in

shaping the bacterial profiles in KCM_2.1, whereas these factors were less significant in KCM_2.2. Similarly, sand content strongly affected bacterial metabolic patterns in KCM_4.1, while TOC was a more dominant factor in KCM_4.2.

The relatively weak influence of HMs on bacterial metabolic profiles may result from long-term microbial adaptation to HM pollution, since our results indicated the presence of HM-tolerant and HM-resistant bacterial phyla/classes in smelter-polluted soils [14]. Microbial adaptation to prolonged HM exposure is commonly driven by mechanisms such as enhanced metal efflux [15], intracellular sequestration [16], and the activation of general stress-response pathways [17]. Functional redundancy within the soil microbiome further stabilizes ecosystem processes, as multiple taxa can perform overlapping metabolic functions, allowing tolerant species to compensate when sensitive groups decline [18]. Additionally, the buffering effects of soil properties might modify HM toxicity [19]. These combined mechanisms likely promote the persistence of bacterial groups with broad metabolic versatility and ecological plasticity, helping maintain functional stability despite HM pollution and explaining the absence of a clear relationship between HM levels and bacterial metabolic activity.

In conclusion, this study demonstrates that HM pollution induces measurable functional shifts within bacterial communities, with the strongest effects observed in soils characterized by adverse physicochemical conditions. The findings indicate that local environmental factors can modulate the expression of HM toxicity, either amplifying or buffering its impact on microbial metabolic processes. Moreover, the persistence of functional activity under long-term HM exposure highlights the adaptive capacity of soil microbiota, although this resilience may depend on specific soil attributes. From an applied perspective, maintaining a near-neutral pH and enhancing inputs of dead organic matter appear to be effective strategies for mitigating the functional impairment of microbial communities in polluted soils. Such measures may help sustain essential soil processes and support ecosystem stability in environments subjected to chronic metal pollution. Despite the limitations of the Biolog test as a culture-dependent method, combining its results with metabolic pathway predictions derived from metagenomic data may provide a more complete and robust evaluation of the metabolic potential of the microbiome in HM-polluted soils in future studies.

REFERENCES

- [1] ZHOU Z., C. WANG, Y. LUO (2020) Meta-analysis of the impacts of global change factors on soil microbial diversity and functionality, *Nat. Commun.*, **11**, 3072, <https://doi.org/10.1038/s41467-020-16881-7>.
- [2] LUO X., C. WU, Y. LIN et al. (2023) Soil heavy metal pollution from Pb/Zn smelting regions in China and the remediation potential of biomineralization, *J.*

- Environ. Sci., **125**, 662–677, <https://doi.org/10.1016/j.jes.2022.01.029>.
- [3] NIKOLOVA R., A. KENAROVA, S. BOTEVA et al. (2024) Diversity and structure of soil bacterial communities in the area of non-ferrous metal plant revealed by 16S rRNA gene retrieval, *C. R. Acad. Bulg. Sci.*, **77**(8), 1260–1268, <https://doi.org/10.7546/CRABS.2024.08.18>.
 - [4] BOTEVA S., A. KENAROVA, V. KANCHEVA et al. (2021) Long-term heavy metal pollution of soils and its impact on bacterial carbon metabolism, *For. Ideas*, **27**(1 (61)), 245–255.
 - [5] LUKHELE T., T. A. M. MSAGATI (2024) Assessment of functional diversity of heterotrophic microbial communities in polluted environments through community level physiological profiles, *Biologia*, **79**, 1027–1039, <https://doi.org/10.1007/s11756-024-01608-6>.
 - [6] JIANG J., R. LIU, Y. DU et al. (2019) Microbial induced carbonate precipitation for immobilizing Pb contaminants: Toxic effects on bacterial activity and immobilization efficiency, *Sci. Total Environ.*, **672**, 722–731, <https://doi.org/10.1016/j.scitotenv.2019.03.294>.
 - [7] MA S., L. QIAO, X. LIU et al. (2022) Microbial community succession in soils under long-term heavy metal stress from community diversity-structure to KEGG function pathways, *Environ. Res.*, **214**(2), 113822, <https://doi.org/10.1016/j.envres.2022.113822>.
 - [8] MARTÍNEZ-TOLEDO Á., D. GONZÁLEZ-MILLE, M. GARCÍA-ARREOLA et al. (2021) Patterns in utilization of carbon sources in soil microbial communities contaminated with mine solid wastes from San Luis Potosi, Mexico, *Ecotoxicol. Environ. Saf.*, **208**, 111493, <https://doi.org/10.1016/j.ecoenv.2020.111493>.
 - [9] CANDAN E. D., N. İDİL, O. CANDAN (2021) The microbial community in a green turtle nesting beach in the Mediterranean: Application of the Biolog EcoPlate approach for beach pollution, *Environ. Sci. Pollut. Res.*, **28**(36), 49685–49696, <https://doi.org/10.1007/s11356-021-14196-8>.
 - [10] NIKOLOVA R., S. BOTEVA, A. KENAROVA et al. (2023) Enzyme activities in soils under heavy metal pollution: a case study from the surroundings of a non-ferrous metal plant in Bulgaria, *Biotechnol. Biotechnol. Equip.*, **37**, 49–57, <https://doi.org/10.1080/13102818.2022.2149348>.
 - [11] KENAROVA A., G. RADEVA, I. TRAYKOV et al. (2014) Community level physiological profiles of bacterial communities inhabiting uranium mining impacted sites, *Ecotoxicol. Environ. Saf.*, **100**, 226–232, <https://doi.org/10.1016/j.ecoenv.2013.11.012>.
 - [12] XIAO Y., M. GUO, X. LI et al. (2020) Spatial distribution, pollution, and health risk assessment of heavy metal in agricultural surface soil for the Guangzhou-Foshan urban zone, South China, *PLoS One*, **15**, e0239563, <https://doi.org/10.1371/journal.pone.0239563>.
 - [13] CHINTHALAPUDI D. P. M., S. POKHREL, W. L. KINGERY et al. (2023) Exploring the synergistic impacts of cover crops and fertilization on soil microbial metabolic diversity in dryland soybean production systems using Biolog EcoPlates, *Appl. Biosci.*, **2**(3), 328–346, <https://doi.org/10.3390/applbiosci2030022>.
 - [14] NIKOLOVA R., E. GATEV, A. KENAROVA et al. (2025) Selective pressure of heavy metals in soil microbial taxa near a smelting area, *Toxics*, **13**(12), 1025, <https://doi.org/10.3390/toxics13121025>.

- [15] NIES D. H. (2003) Efflux-mediated heavy metal resistance in prokaryotes, *FEMS Microbiol. Rev.*, **27**(2-3), 313–339, [https://doi.org/10.1016/S0168-6445\(03\)00048-2](https://doi.org/10.1016/S0168-6445(03)00048-2).
- [16] BLINDAUER C. A., O. I. LESZCZYSZYN (2010) Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more, *Nat. Prod. Rep.*, **27**(5), 720–741, <https://doi.org/10.1039/b906685n>.
- [17] BARAL R., K. HO, R. P. KUMAR et al. (2024) A general mechanism for initiating the general stress response in bacteria, *eLife*, **13**, RP100376, <https://doi.org/10.7554/eLife.100376.2>.
- [18] LOUCA S., M. F. POLZ, F. MAZEL et al. (2018) Function and functional redundancy in microbial systems, *Nat. Ecol. Evol.*, **2**, 936–943, <https://doi.org/10.1038/s41559-018-0519-1>.
- [19] KHAFIDA W., B. KLIMEK, M. NIKLICSKA (2024) The relationship between soil bacteria carbon utilization and soil physicochemical properties, *E3S Web Conf.*, **495**, 02006, <https://doi.org/10.1051/e3sconf/202449502006>.

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