Differential Effect of Monoterpenes and Flavonoids on the Transcription of Aromatic Ring-Hydroxylating Dioxygenase Genes in *Rhodococcus opacus* C1 and *Rhodococcus* sp. WAY2

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Supplementary material

Genome analysis of strain C1

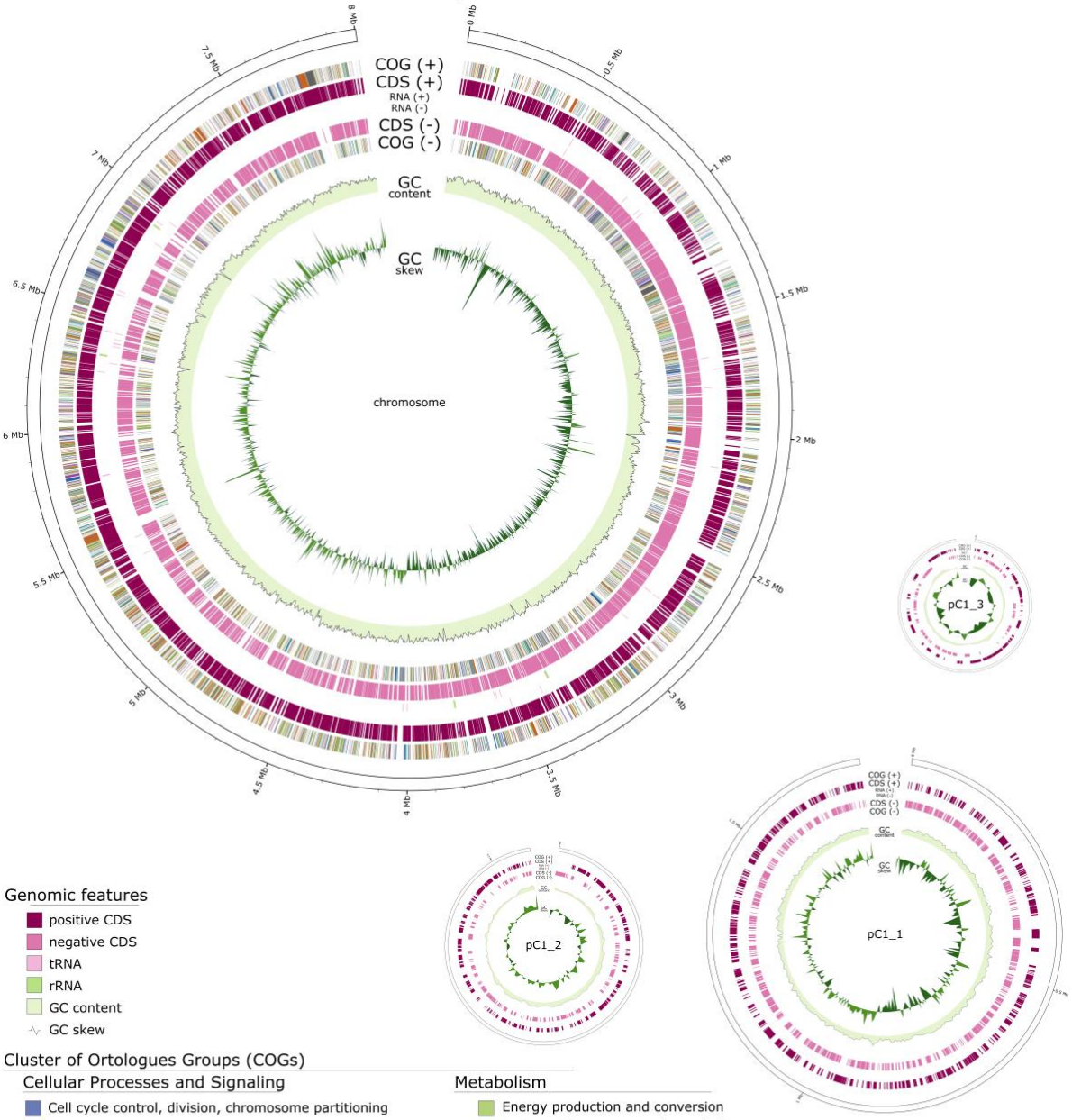
The culture was grown overnight on PCA at 28°C. Genomic DNA was isolated with a FastDNA SPIN Kit (MPBio, USA) following the manufacturer's protocol. Sequencing libraries were prepared using rapid sequencing kit (Oxford Nanopore) and sequenced on a Nanopore MinION paltform (FLO-MIN106). In total, 578 328 Nanopore reads (average read length, 4 729.5 bp; the total number of bases, 2.7 Gbp) were generated, corresponding approximately to a 47× genome coverage. The sequencing adaptors were removed using porechop (v.0.2.4) and the reads were further filtered out using Filtlong (v.0.2.1) with options --min_length 1000 --keep_percent 90. The cleaned reads were assembled using Flye (v.2.9.2) [1] with options --nano-corr. The obtained assembly was polished with Medaka (v.1.11.3) with option -m r941_min_hac_g507. The data were submitted to NCBI GenBank under the Bioproject PRJNA1028948.

Genome characteristics

The genome of *Rhodococcus opacus* C1 was obtained with 94.71% completeness and 3.55% redundancy as measured by CheckM (v.1.2.3) [3]. The genome consists of one linear chromosome (8,002,894 bp) with three linear plasmids (1,727,788 bp, 536,566 bp, and 244,600 bp) with an average GC-content of 67% (Fig. S1). The PGAP annotation [4] identified 722 pseudogenes of which 462 were frameshifted, 382 were incomplete, and 49 had internal stop codons. A total of 9,843 genes were identified, including 51 tRNAs and 12 rRNAs (4 5S, 4 16S and 4 23S). Genome maps were generated using PGAP annotation in the GenoVi tool [2]. Taxonomy placement was performed using GTDBtk (v.2.3.0) [5], which identified the closest genome as that of *Rhodococcus opacus* $1b^T$ with 99.16% ANI.

Supplementary Figure S1. Genomic map of *Rhodococcus opacus* C1 replicons [2]. The linear chromosome (CP137577, 67.5 % GC content) and three linear plasmids pC1_1 (CP137575, 65.5 % GC content), pC1_2 (CP137576, 64.5 % GC content), and pC1_3 (CP137574, 65 % GC content) are displayed. The outer two rings represent coding sequences in the forward strands (CDS+, second ring) colored according to the COG (clusters of orthologous groups, first ring) types. Other genomic features, such as tRNA and rRNA, are displayed in the rings below. The inner two rings represent a deviation in the G+C content and G+C skew.

Rhodococcus opacus C1



Search for ARHDs

To obtain a complete set of ARHDs in *Rhodococcus opacus* C1 (Table S1), both the ARHD α-subunit and Rieske component HMM profiles (available at UniProt: PF00848.23, PF00355.30 respectively) were simultaneously searched against the C1 genome with HMMER (v.3.4) [6]. Putative functions of the ARHD sequences were then searched using BLASTP against the nr database.

Table S1. Set of ARHDs in *Rhodococcus opacus* C1. ARHD α-subunit and Rieske component HMM profiles were searched against the C1 genome with HMMER. Putative protein specificity was searched by BLAST against nr database.

Gene Replicon: gene ID (gene coordinates)	**Identity [%]**	**Accession Number**	**Putative Protein Specificity**	**Organism**	**Citation**
nahA1 CP137574: R1X32_00130 (23,220-24,626)	98.93	WP_159927768.1	Naphthalene dioxygenase large subunit	*Rhodococcus* sp. WAY2	[7]
bphA1 CP137575: R1X32_08185 (1,490,248-1,491,630)	99.35	WP_011599002.1	Biphenyl 2,3-dioxygenase subunit alpha	*Rhodococcus jostii* RHA1	[8]
CP137575: R1X32_01475 (78,486-79,832)	100	WKN61298.1	ARHD	*Rhodococcus opacus 3*D	[9]
cmtA1 CP137575: R1X32_05705 (977,693-979,006)	97.71	TQC41472.1	*p*-cumate dioxygenase	*Rhodococcus* sp. WS4	x
CP137576: R1X32_11090 (366,323-367,660)	99.2	WP_317571927.1	x	x	x
CP137576: R1X32_11620 (501,838-503,016)	55.95	HWI71024.1	ARHD	*Beakduia* sp.	[10]
phtA1 CP137577: R1X32_33330 (4646,017-4647,462)	98.75	AAR90178.1	phthalate 3,4-dioxygenase subunit alpha	*Rhodococcus* sp. DK17	[11]
tphA1 CP137577: R1X32_33285 (4,637,988-4,639,253)	99.76	ELB91386.1	terephthalate 1,2-dioxygenase alpha subunit	*Rhodococcus wratislaviensis* IFP 2016	[12]
CP137577: R1X32_34835 (4,977,473-4,978,762)	100	WP_005574578.1	x	x	x
benA1 CP137577: R1X32_36710 (5,386,709-5,388,085)	100	WP_120660447.1	benzoate 1,2-dioxygenase large subunit	x	x
CP137577: R1X32_39665 (6,006,694-6,007,866)	100	WP_007300410.1	x	x	x

Utilization of naphthalene and other SPMs by strain C1

Strain C1 was tested for its ability to use a wider range of SPMs and naphthalene as the sole carbon source at a concentration of 1 mM (Tab. S2). Samples were prepared as described in the Materials and Methods section.

Strain C1 was able to utilize naphthalene and most of the tested phenolic compounds, except for 4-hydroxycoumaric acid, salicylic acid, and vanillin (Tab. S2).

Table S2. SPMs and naphthalene utilization by strain C1.

Group of SPMs	Substrate	Strain C1
	Naphthalene	U
Flavonoids	Morin	-
	Chrysin	-
Coumarins	Umbelliferon	-
Phenolic acids	Caffeic acid	U
	Cinnamic acid	U
	Ferulic acid	U
	Gallic acid	U
	p-hydroxybenzoic acid	U
	4-hydroxycoumarin	-
	p-coumaric acid	U
	Protocatechuic acid	U
	Salicylic acid	-
	Vanillin	-

Transformation of SPMs and biphenyl by strains C1 and WAY2

Analysis of flavonoids

High-performance liquid chromatography (HPLC) coupled with a UV-VIS diode array detector was used to analyze flavonoids: flavone, luteolin, flavanone, naringenin, kaempferol, quercetin, catechin, daidzein, and genistein. The analysis was conducted using the NexeraXR system equipped with the SPD-M20A detector (Shimadzu). Prior to the analysis, an equal volume of methanol was added to the glass vials, cell suspensions were sonicated for 15 min, and cell debris was removed by centrifugation. The content of individual flavonoids in the samples was then determined with the HPLC, using the following program: 0-1.2 min – 20% A, 1.5-4 min – gradient of 20%-80% A, 3.5-5.5 min – 95% A, 4-7 min – 80%, 7-8 min gradient of 80% – 20% A, 80-10 min – 20% A; mobile phase A: methanol with 0.02% (v/v) trifluoracetic acid, B: H2O with 0.02% (v/v) trifluoracetic acid; mobile phase flow 0.8 ml/min. A Luna® Omega Polar C18 column (Phenomenex) was used for separation at 40 °C. The detection wavelengths [nm] and corresponding elution times [min] for each compound were the following: flavone 296/7.13, luteolin 366/6.18, flavanone 249/7.00, naringenin 289/6.08 kaempferol 262/6.31, quercetin 370/6.01, catechin 220/2.64, daidzein 248/5.94, genistein 259/6.19. The linearity of the detector response was verified for the concentration range of 1 – 500 µM for all analytes.

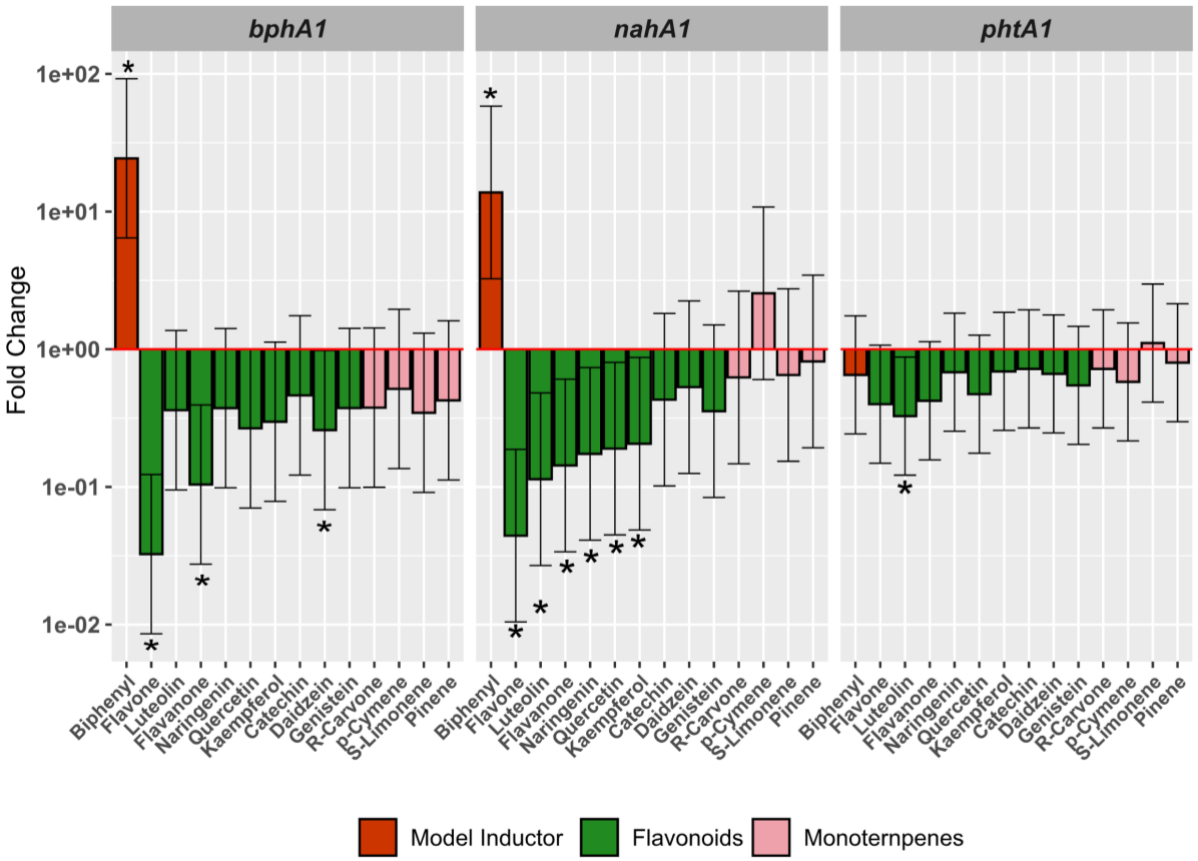
Analysis of monoterpenes

Gas chromatography coupled with mass detection (GC-MS) was employed to analyze monoterpenes, including (*S*)-limonene, (*R*)-carvone, *p*-cymene, and *α*-pinene. The analysis was conducted using a Varian 450-GC instrument (USA) with a Combi-Pal autosampler (CTC Analytics, Sweden) and a Varian 240 MS ion trap mass spectrometer. Prior to the analysis, samples were extracted with ethyl acetate (5 ml; 3 times) employing a reciprocating shaker. The extracts were dried with sodium sulfate, concentrated to 1 ml using a nitrogen stream, and analyzed by GC-MS with a sample injection volume of 1 µl. Separation was carried out on a DB-5MS column (Agilent, Germany), which was 30 mm long x 0.25 mm I.D. with a 0.25 µm film thickness. The GC oven temperature program started at 45 °C (held for 6 min), then gradually increased to 80 °C (2 °C/min, held for 5 min), and finally heated up to 200 °C (10 °C/min), where it was held isothermally for 2 min. The GC oven temperature program for the analysis began at 60°C (held for 1 minute) and gradually increased to 280°C (15°C/min, held for 10 minutes). Helium (99.999%) served as the carrier gas at a flow rate of 1 ml/min. The injector temperature was set to 240°C. The electron impact ion source, ion trap, and transfer line temperatures were set to 250°C, 220°C, and 280°C, respectively. The mass spectra were recorded at 3 scans/s at 70 eV over the mass range of 50-300 amu.

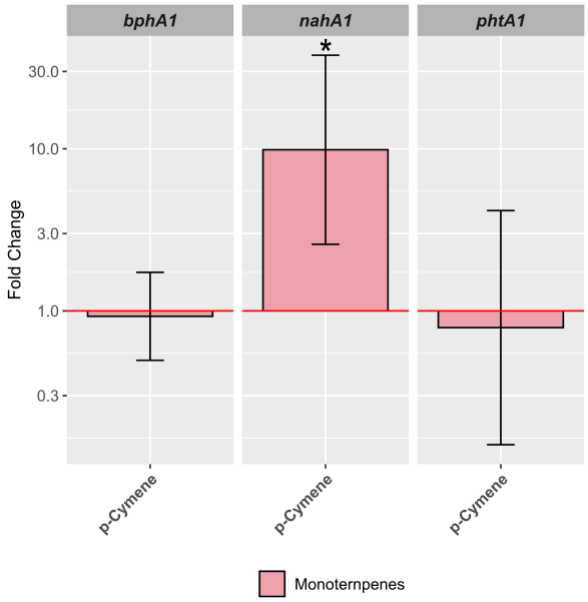
Transcription of ARHDs genes in strains C1 and WAY2 in the presence of SPMs and biphenyl

To assess the ability of the SPMs to regulate the transcription of the ARHD genes in strains C1 and WAY2, relative transcription of ARHD genes was evaluated at the mRNA level after previous exposure of both strains to SPMs.

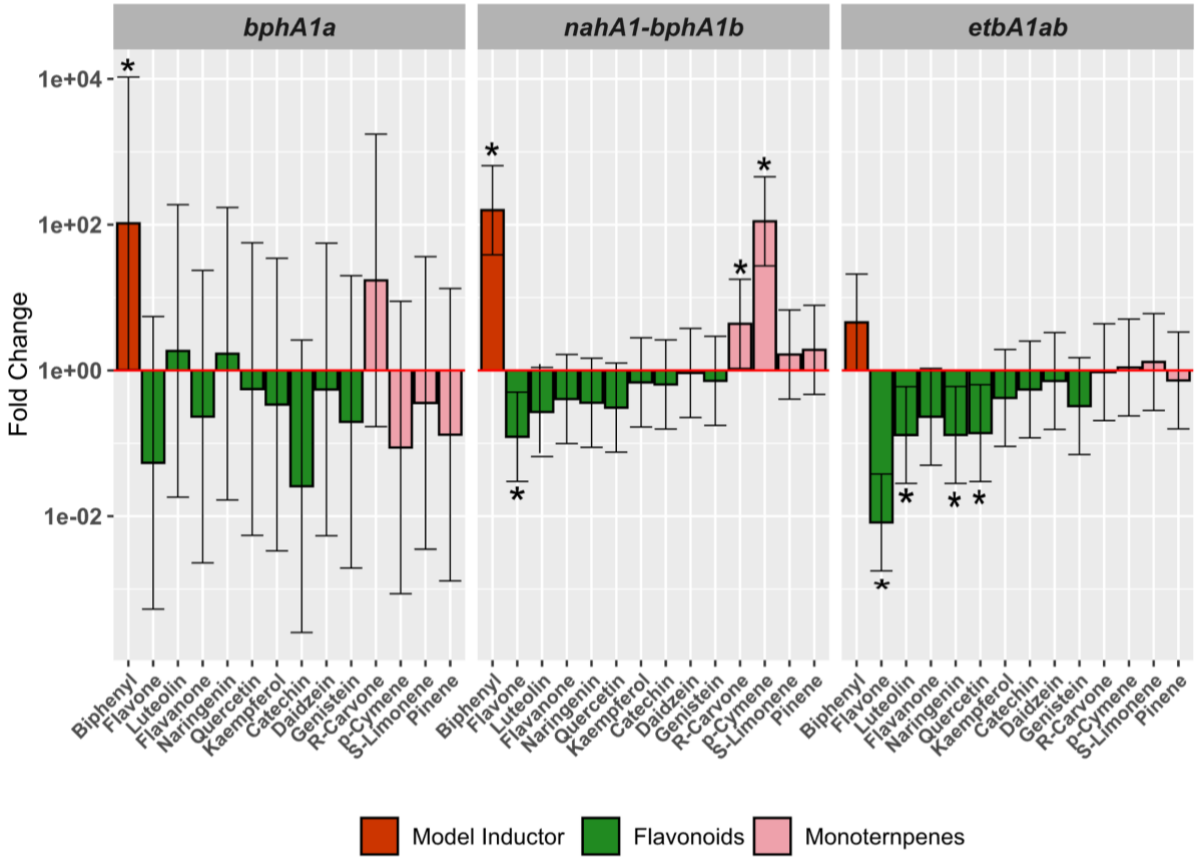
Supplementary Figure S2. Relative transcription of *bphA1, nahA1*, and *phtA1* genes in strain C1 after incubation with SPMs and biphenyl for 6h. Samples were prepared in triplicates. The transcription levels were processed by the Common Based Method [13]. Data were normalized to the transcribed levels of the 16S rRNA and *gyrB* genes, analyzed by ANOVA (α = 0.05) followed by the Dunnett post-hoc test and presented with 95% confidence intervals. An asterisk indicates a significant difference in the relative level of gene transcripts compared to the unaffected cells, i.e. control.



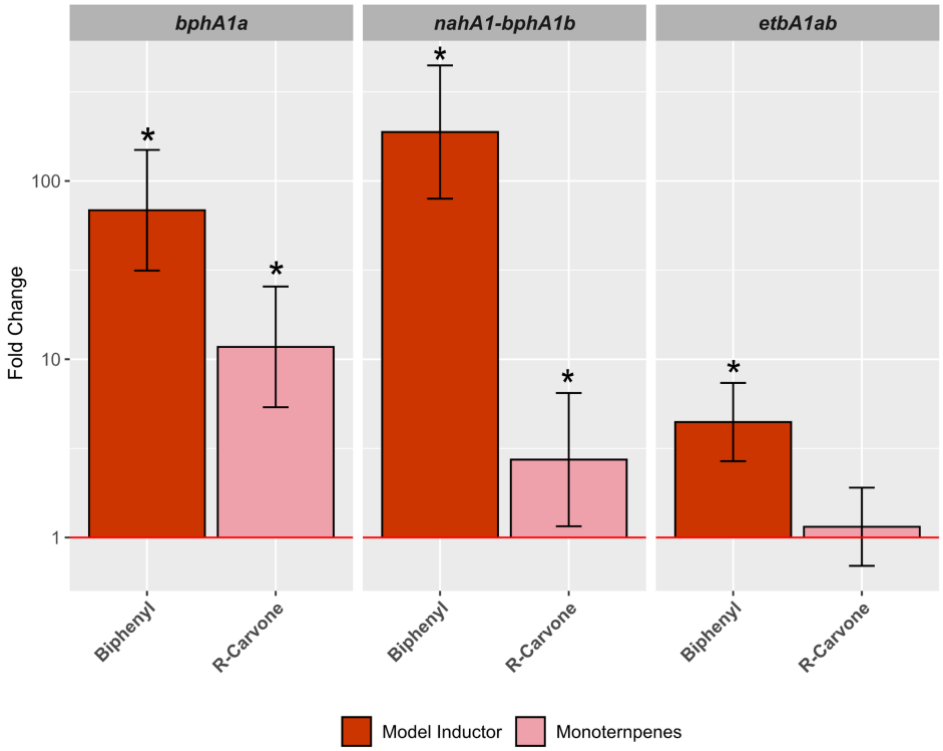
Supplementary Figure S3. Relative transcription of *bphA1, nahA1*, and *phtA1* genes in strain C1 after incubation with *p*-cymene for 6h. Samples were prepared in 7 replicates. The transcription levels were processed by the Common Based Method [13]. Data were normalized to the transcribed levels of the 16S rRNA and *gyrB* genes, analyzed by t-test (α = 0.05), and are presented with 95% confidence intervals. An asterisk indicates a significant difference in the relative level of gene transcripts compared to the unaffected cells, i.e. control.



Supplementary Figure S4. Relative transcription of *bphA1a*, *nahA1-bphA1b*, and *etbA1ab* genes in strain WAY2 after incubation with SPMs and biphenyl for 3h. Samples were prepared in triplicates. The transcription levels were processed by the Common Based Method [13]. Data were normalized to the transcribed levels of the 16S rRNA and *gyrB* genes, analyzed by ANOVA (α = 0.05) followed by the Dunnett post-hoc test, and are presented with 95% confidence intervals. An asterisk indicates a significant difference in the relative level of gene transcripts compared to the unaffected cells, i.e. control.



Supplementary Figure S5. Relative transcription of *bphA1a, nahA1-bphA1b,* and *etbA1ab* genes in strain WAY2 after incubation with *R*-carvone and biphenyl for 3h. Samples were prepared in 7 replicates. The transcription levels were processed by the Common Based Method [13]. Data were normalized to the transcribed levels of the 16S rRNA and *gyrB* genes, analyzed by t-test (α = 0.05), and are presented with 95% confidence intervals. An asterisk indicates a significant difference in the relative level of gene transcripts compared to the unaffected cells, i.e. control.



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