Diesel exhaust particles alter gut microbiome and gene expression in the bumblebee Bombus terrestris

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Abstract

Insect decline is a major threat for ecosystems around the world as they provide many important functions, such as pollination or pest control. Pollution is one of the main reasons for the decline, besides changes in land use, global warming, and invasive species. While negative impacts of pesticides are well studied, there is still a lack of knowledge about the effects of other anthropogenic pollutants, such as airborne particulate matter, on insects. To address this, we exposed workers of the bumblebee Bombus terrestris to sublethal doses of diesel exhaust particles (DEPs) and brake dust, orally or via air. After seven days, we looked at the composition of the gut microbiome and tracked changes in gene expression. While there were no changes in the other treatments, oral DEP exposure significantly altered the structure of the gut microbiome. In particular, the core bacterium Snodgrassella had a decreased abundance in the DEP treatment. Similarly, transcriptome analysis revealed changes in gene expression after oral DEP exposure, but not in the other treatments. The changes are related to metabolism and signal transduction which indicates a general stress response. Taken together, our results suggest potential health effects of DEP exposure on insects, here shown in bumblebees, as gut dysbiosis may increase the susceptibility of bumblebees to pathogens, while a general stress response may lower available energy resources. However, experiments with multiple stressors and on colony level are needed to provide a more comprehensive understanding of the impact of DEPs on insects.

Research article

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Abstract

Insect decline is a major threat for ecosystems around the world as they provide many important functions, such as pollination or pest control. Pollution is one of the main reasons for the decline, besides changes in land use, global warming, and invasive species. While negative impacts of pesticides are well studied, there is still a lack of knowledge about the effects of other anthropogenic pollutants, such as airborne particulate matter, on insects. To address this, we exposed workers of the bumblebee *Bombus terrestris* to sublethal doses of diesel exhaust particles (DEPs) and brake dust, orally or via air. After seven days, we looked at the composition of the gut microbiome and tracked changes in gene expression. While there were no changes in the other treatments, oral DEP exposure significantly altered the structure of the gut microbiome. In particular, the core bacterium *Snodgrassella*had a decreased abundance in the DEP treatment. Similarly, transcriptome analysis revealed changes in gene expression after oral DEP exposure, but not in the other treatments. The changes are related to metabolism and signal transduction which indicates a general stress response. Taken together, our results suggest potential health effects of DEP exposure on insects, here shown in bumblebees, as gut dysbiosis may increase the susceptibility of bumblebees to pathogens, while a general stress response may lower available energy resources. However, experiments with multiple stressors and on colony level are needed to provide a more comprehensive understanding of the impact of DEPs on insects.

Introduction

Global biodiversity loss is one of the major challenges humanity currently faces (Diaz et al. 2006, Dirzo et al. 2014). Especially the rapid decline in insects is cause for concern, as they provide or contribute to many important ecosystem functions such as pollination, nutrient cycling, pest control, and linking trophic levels (Cardoso et al. 2020, Noriega et al. 2018). Pollution is one of the major reasons for the decline besides intensification of land use, climate change, and invasive species (Milicic et al. 2021, Sanchez-Bayo & Wyckhuys 2019).

Pesticides harm insects on many different levels ranging from subtle changes in the gut microbiome over behavioral changes to increased mortality (Desneux et al. 2007, Motta et al. 2018, Ndakidemi et al. 2016). Other anthropogenic pollutants might also contribute to the observed declines in insects, but their impacts are often less well studied (Cameron & Sadd 2020, Feldhaar & Otti 2020, Sanchez-Bayo & Wyckhuys 2019). Airborne particulate matter deriving from traffic or industrial processes has become ubiquitous in the environment (Gieré & Querol 2010, Zereini & Wisemann 2010). While the harmful effects on mammals, in particular humans, have been intensively studied, research investigating the impact on insects remains scarce (Kim et al. 2015, Valavanidis et al. 2008). Insects can encounter these pollutants in various ways, e.g. by foraging in contaminated areas, consuming contaminated food or direct deposition on the insect's cuticle (Feldhaar & Otti 2020, Lukowski et al. 2018, Negri et al. 2015). The airborne particulate matter might enter an insect´s body via oral ingestion or the tracheal system (Feldhaar & Otti 2020, Negri et al. 2015). Social insects might be at an increased risk, as pollutants are transferred to and stored in their nests, which could lead to a higher exposure to conspecifics and the brood (Feldhaar & Otti 2020, Hladun et al. 2016).

Vehicle brake dust and diesel exhaust particles (DEPs) are major classes of airborne particulate matter deriving from traffic released into the environment (Hamilton & Hartnet 2013, Harrison et al. 2012, Rönkkö & Timonen 2019). Brake dust particles contain various metals and phenolic compounds, depending on the brake lining used (Iijima et al. 2007, Thorpe & Harrison 2008). Exposure of different invertebrate species to

such particles showed mixed effects. Particulate matter contamination in soil did not affect colony founding in the ant *Lasius niger*(Seidenath et al. 2021). However, soil-feeding earthworms (*Eisenia fetida*) showed a strongly increased mortality when exposed to soil spiked with brake dust particles (Holzinger et al. 2022). DEPs have a different composition than brake dust. They are composed of an elemental carbon core with adsorbed organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), and traces of metals and other elements (Greim 2019, Wichmann 2007). Exposure to high doses of diesel exhaust particles (up to a concentration of 2 g/L) in food over a period of seven days reduced survival in *Bombus terrestris*workers compared to controls (Hüftlein et al. unpublished).

Many classical ecotoxicology approaches focus on the effect of a substance on mortality, growth, or reproduction. However, pollutants can also have more subtle sublethal effects on insects which may have severe consequences in the long-term (Straub et al. 2020). Direct sublethal effects include changes in physiology such as stress reactions or detoxification processes. By interacting with microorganisms inside the insect's body, oral exposure to pollutants may indirectly affect insect health.

Most eukaryotic organisms and their associated microbes form an entity, the so-called holobiont (Theis et al. 2016, Zilber-Rosenberg & Rosenberg 2008). In insects, microorganisms can be found in the digestive tract, the exoskeleton, the hemocoel, or within cells (Douglas 2015). The insect gut microbiome has a range of functions that include protection from pathogens, detoxification, digestion, and the production of essential nutrients (Engel & Moran 2013). Social bumblebees (*Bombus spp.)* and honeybees (*Apis mellifera*) are model organisms to study gut microbiota as their gut microbiome is rather simple and highly conserved (Engel et al. 2016, Kwong & Moran 2016, Zhang & Zheng 2022). A few core bacterial taxa dominate the gut microbiome of bumblebees: *Snodgrassella* , *Gilliamella* ,*Schmidhempelia* , Bifidobacteriaceae (*Bifidobacterium and Bombiscardovia*) and two clusters within Lactobacillaceae (Hammer et al. 2021, Koch & Schmid-Hempel 2011a, Martinson et al. 2011). While many functions of the bacterial symbionts in bumblebees have been proposed, only very few have been demonstrated in experiments (Hammer 2021, Zhang & Zheng 2022). Resistance to the common trypanosomatid parasite*Crithidia bombi* is higher in bumblebees with an intact microbiome compared to microbiota-free individuals (Koch & Schmid-Hempel 2011b). Moreover, infection outcomes of *C. bombi* vary with host microbiota composition rather than genotype (Koch & Schmid-Hempel 2012). The gut microbiome of bumblebees may be important for detoxification as microbiota-free individuals had lower survival when exposed to toxic concentrations of selenate (Rothman et al. 2019).

Examining the effects of anthropogenic pollutants, such as airborne particulate matter, on the gut microbiome is an important tool for assessing their risk for insect health (Duperron et al. 2020). Even with a conserved gut microbiome, the relative abundance of core bacteria and the presence of other microorganisms will vary with age, diet and changing environmental parameters (Kwong & Moran 2016, Koch et al. 2012). Different pollutants affect the microbial composition of bee guts. In honeybee workers, pesticides or antibiotics change the relative and absolute abundance of core gut microbiota species (DeGrandi-Hoffmann et al. 2017, Motta et al. 2018, Raymann et al. 2017). An array of environmental toxicants, such as cadmium, copper, selenate, and hydrogen peroxide, alter the gut microbiome of *Bombus impatiens* at field-realistic concentrations (Rothman et al. 2020). These shifts in the microbial community may affect bumblebee health. Intestinal dysbiosis, compositional and functional alteration of the microbiome, is associated with various diseases and health problems in humans and vertebrates (De Gruttola et al. 2016, Levy et al. 2017, Shreiner et al. 2015). In insects, dysbiosis negatively affects reproductive fitness, immunity, and resistance to pathogens (Ami et al. 2010, Daisley et al. 2020, Raymann et al. 2017).

Transcriptome analysis is a sensitive tool to characterize sublethal effects of potentially harmful substances on a molecular and cellular level (Prat & Degli-Esposti 2019, Schirmer et al. 2010). Changes in gene expression help to identify biological processes, such as stress responses and detoxification processes, at an early stage. Exposure to different pollutants have been shown to induce changes in gene expression in several insect species. Mosquitos (*Aedes aegypti*) exposed to anthropogenic pollutants (insecticides, PAHs) increased the expression of genes related to detoxification, respiration and cuticular proteins (David et al. 2010). Fireflies (*Luciola leii*) showed a similar response when exposed to benzo(a)pyrene, a widespread PAH (Zhang et

al. 2019). In different bee species, the neonicotinoids imidacloprid, thiamethoxan, and clothianidin induce an upregulation of metabolic, immune and stress response genes (Aufauvre et al. 2014, Bebane et al. 2019, Christen et al. 2018, Colgan et al. 2019, Gao et al. 2020, Shi et al. 2017). The expression of genes related to detoxification was higher in honeybees (*A. mellifera*) exposed to heavy metals than in controls (Al Naggar et al. 2020, Gizaw et al. 2020, Zhang et al. 2018).

In contrast to pesticides, the effects of other environmental pollutants, such as particulate matter, on gene expression in bees as well as their gut microbiome are largely unclear. To address this knowledge gap, we exposed workers of the buff-tailed bumblebee*Bombus terrestris* to airborne particulate matter deriving from traffic and investigated changes in the gut microbiome and gene expression. Bumblebees were fed sugar water spiked with sublethal concentrations of brake dust or diesel exhaust particles (DEPs). Adding to this oral exposure, one group of bumblebees was exposed to DEPs via air to enable potential uptake in the tracheal system. We expect changes in the composition of the gut microbial community, as previous research showed changes due to different metals in a closely related*Bombus* species (Rothman et al. 2020). Moreover, we expect changes in the expression of detoxification and metabolic genes, indicating an increased stress level, as the toxic compounds in the particulate matter may interfere with bumblebee physiology.

Methods

Bumblebee keeping

Four queenright colonies of *B. terrestris* were ordered from Biobest (Westerlo, Belgium) in March 2021. Colonies were kept in a climate chamber at 26° C and 70 % humidity under a constant, inverted 12:12 h light:dark cycle. Colonies were provided with sugar water (50% Apiinvert, Südzucker AG, Mannheim, Germany) and pollen (Imkerpur, Osnabrück, Germany) *ad libitum* .

Experimental procedure

At the beginning of the experiment, adult workers from the four colonies were randomly assigned to one of six treatments. **Control** : fed with sugar water only (50% Apiinvert) (n=56); **Solvent control** : fed with sugar water spiked with 0.02% (v/v) of the emulsifier Tween20 (n=56); **Brake dust** : fed with sugar water spiked with 0.02% (v/v) of the emulsifier Tween20 and 0.4 g/l brake dust particles (n=56);**DEP** : fed with sugar water spiked with 0.02% (v/v) of the emulsifier Tween20 and 0.4 g/l diesel exhaust particles (n=56); **Flight control** : fed with sugar water (50% Apiinvert) and allowed to fly once per day in a plastic box (7 x 7 x 5 cm, EMSA, Emsdetten, Germany) for 3 minutes (n=24); **DEP flight** : fed with sugar water (50% Apiinvert) and allowed to fly once per day for 3 minutes in a plastic box (7 x 7 x 5 cm, EMSA, Emsdetten, Germany) that contained 1.5 (+-0.1) mg of diesel exhaust particles (n=24).

The experiment was conducted in a climate chamber at 26° C and 70 % humidity under a constant 12:12 h light:dark cycle. Bumblebees were kept in Nicot cages (Nicotplast SAS, Maisod, France) connected to a 12 ml syringe (B. Braun SE, Melsungen, Germany) with the tip cut off, that contained 2 ml of the respective feeding solution (*ad libitum*). Every day the syringes were replaced with fresh ones to prevent molding or bacterial growth in the food. The exposure lasted for seven days. At the end of the experiment, the animals were frozen at -20° C.

Within a week after the end of the experiment, we randomly selected twelve (three workers per colony) bumblebees per treatment for transcriptome analysis (N=72). Additionally, for the control, solvent control, brake dust and DEP treatment, we randomly selected 20 bumblebees (five workers per colony) for microbiome analysis (N=80), respectively.

Generation and collection of diesel exhaust particles (DEPs)

DEPs were collected from a four-cylinder diesel engine (OM 651, Daimler AG, Stuttgart, Germany) during a repeating cycle of transient and stationary operating points, resembling an inner-city driving scenario with stop-and-go intervals. The engine was operated on a test bench with a water-cooled eddy-current brake as previously described in Zöllner (2019). DEP samples were collected by an electrostatic precipitator

(OekoTube Inside, Mels-Plons, Switzerland). A fast response differential mobility particulate spectrometer DMS500 (Combustion, Cambridge, England) was applied to measure sub-micron particle size distributions of raw exhaust samples. Depending on engine load and speed during the inner-city cycle, solid particles showed a median diameter between 52.1 ± 1.8 nm and 101.9 ± 1.7 nm. DEP composition was characterized by thermogravimetric analysis (TGA, STA 449 F5 Jupiter, Netzsch-Gerätebau GmbH, Selb, Germany). A fraction of 72.2 ± 1.1 % of the DEP mass was attributed to elemental carbon, 23.2 ± 0.9 % w/w to organic fractions and 4.6 ± 0.7 % w/w to inorganic matter. Quantification of PAHs revealed concentrations of 444 ppm for pyrene, 220 ppm for phenanthrene, and 107 ppm for fluoranthene.

The elemental composition of the DEP samples was analyzed by Inductively-Coupled Plasma Optical Emission Spectrometry (ICP-OES, Optima 7300 DV, PerkinElmer Inc., Waltham, United States of America) and interpreted according to Zöllner (2019). It showed fractions of calcium (1.63 % w/w), zinc (0.53 % w/w) and phosphorus (0.50 % w/w) that can be traced back to diesel fuel and lubrication oil. Copper (1.03 % w/w), aluminum (0.02 % w/w) and iron (0.02 % w/w) can be attributed to abrasion of piston rings, cylinder head and engine block material, respectively. In addition, small amounts of boron (0.13 % w/w), magnesium (0.10 % w/w), molybdenum (0.03 % w/w), natrium (0.02 % w/w) and sulphur (0.17 % w/w) were found.

Generation of brake dust particles

The brake dust particles provided by the Chair of Ceramic Materials Engineering of the University of Bayreuth are derived by LowMet brake pads (provided by TMD Friction Holdings GmbH, Leverkusen, Germany) that were milled for three minutes in a vibrating cup mill with a tungsten carbide grinding set (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany). LowMet brake pads are common and representative for passenger cars in Europe and consist of non-ferrous metals (25 % (w/w)), steel wool (15 % (w/w)), petrol coke (12 % (w/w)), sulphides (10 % (w/w)), aluminum oxide (5 % (w/w)), resin (5 % (w/w)), graphite (4 % (w/w)), mica (4 % (w/w)), silicon carbide (3 % (w/w)), barite (2 % (w/w)), fibers (2 % (w/w)), and rubber (1 % (w/w)) (Wiaterek 2012). The particle size distribution of the milled, fine-grained powder was measured with a laser diffraction particle size analyzer (PSA 1190 LD, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany). The mean particle size found was 10.19 ± 4.37 µm (D10 = 0.68 µm (10% of all particles being smaller in diameter than this size), D50 = 5.76 µm (median particle size), D90 = 25.87 µm (90% of particles being smaller in diameter than this size)).

Bumblebee gut microbiome analysis

Prior to dissection bumblebees were defrosted and rinsed in 70% ethanol, 90% ethanol and twice in ultrapure water. We placed each bumblebee on an autoclaved square of aluminum foil (5 x 5 cm) and opened the abdomen with sterilized tweezers and scissor. After carefully separating the gut from the crop and transferring it to an Eppendorf tube, we snap-froze the gut in liquid nitrogen. All samples were stored at -80° C until further processing.

PCR amplification and sequencing of 16S rDNA fragments

Metagenomic DNA of bumblebee gut samples was purified using the NucleoMag DNA Bacteria kit (Macherey-Nagel, no. 744310, Düren, Germany) after disruption of samples with 1.4 mm (diam.) ceramic beads (no. P000912-LYSK0A, Bertin Instruments, Montigny-le-Bretonneux, France) in a FastPrep-24 bead beating device (MPbio, Irvine, USA) following the instructions of the manufacturer. The metagenomic DNA was diluted to a concentration of 5 ng/µl, and 2.5 µl DNA was used to amplify 16S rDNA fragments using primers 515F-Y (Turner et al. 1999) and 806RB (Apprill et al. 2015) as described in the 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B, www.illumina.com). Sample libraries were barcoded using the Nextera XT index kit (v2 set A, www.illumina.com), combined in equimolar amounts, and sequenced on Illumina's iSeq-100 platform using a 293 cycle single-end R1 mode. Demultiplexing of reads was performed by the iSeq-100 local run manager and sample-specific reads were saved in FastQ format.

Microbiome analysis

Statistical analyses of the microbial data were performed using QIIME2 (Bolyen et al. 2019) and R 4.2.1 (R Core Team 2022). Forward reads of 16S rDNA fragments (R1 reads) were analyzed using the QIIME2 microbiome analysis package (ver. 2021.11; Bolyen et al. 2019). Unless indicated otherwise, all analysis tools were used as plugins of the QIIME2 package. The respective parameters used along the analysis steps are readily accessible by provenance information in the QIIME2 data files (available as supplemental data). In brief, the following analysis steps were performed: Demultiplexed reads were trimmed for 16S primer sequences (plugin cutadapt; Martin 2011), denoised, dereplicated and chimera-checked (plugin DADA2; Callahan et al. 2016) resulting in amplified sequence variants (ASVs). Rare ASVs were filtered using the median frequency of ASVs over all samples. Taxonomic classification of ASVs was performed (plugin feature-classifier; Bokulich et al. 2018) using the pre-fitted sklearn-based taxonomy classifiers based on the SILVA reference database (ver. 138.1; (Quast et al. 2013; Yilmaz et al. 2014). ASVs that could not be taxonomically assigned at any taxonomic level ('unassigned') as well as samples with less than 3,900 reads in total were removed prior to subsequent analysis steps. Alpha diversity metrics, such as Shannon diversity index, Faith's phylogenetic diversity, Pielou's eveness, and observed ASVs, were obtained using the QIIME2's 'core-metrics-phylogenetic' workflow (plugin diversity), rarefied to 3,900 reads per sample. To find significant differences in α-diversity we fitted generalized linear models (GLMs) with treatment as factor. We checked model assumptions using model diagnostic test plots, i.e., qqplot and residual vs. predicted plot from the package*DHARMa* (Hartig 2022). Depending on model assumptions, we then used Kruskal-Wallis tests or produced F-statistics with the function*Anova* () from the package *car* (Fox & Weisberg 2019) to calculate p-values for differences between treatments. For significant treatment effects, we ran pairwise comparisons. In the case of a significant Kruskal-Wallis test, pairwise comparisons were done using Dunn's test for multiple comparisons with Benjamini-Hochberg correction (package *dunn.test* (Dinno 2007)). In the case of a significant ANOVA, pairwise comparisons were made using Tukey HSD post-hoc test with Benjamini-Hochberg correction from the package *multcomp* (Hothorn et al. 2008). Differential abundance of the rarefied data we analyzed using the package *DESeq2* with a negative binomial distribution, a significance level cutoff of FDR < 0.01, replacement of outliers turned off, and cooksCutoff turned off (Love et al. 2014). Compositional differential abundance analysis was performed using Aldex2 (plugin aldex2; Fernandes et al. 2013). Beta diversity of the sparse, compositional microbiome data were calculated using QIIME2's plugin DEICODE which performs a robust Aitchison PCA (Martino et al. 2019). Significance was tested in a PERMANOVA with 999 permutations followed by pairwise PERMANOVA with Benjamini-Hochberg (BH) correction for multiple testing (Anderson 2008). We used the packages *qiime2R* (Bisanz 2018) and *mia* (Ernst et al. 2022a) to import and process the microbiome data in R. Data were arranged using the package *tidyr*(Wickham & Girlich 2022) and were plotted using the packages*ggplot2* (Wickham 2016), *ggpubr* (Kassambra 2020), and*miaViz* (Ernst et al. 2022b).

Transcriptome analysis of whole bumblebee abdomens

Bumblebees were defrosted and rinsed in 70% ethanol, 90% ethanol and twice in ultrapure water prior to dissection. The abdomen was cut off with sterile scissors, placed in an Eppendorf tube and snap-frozen in liquid nitrogen. All samples were stored at -80° C until further processing.

RNA sequencing

Total RNA was prepared from abdomen samples using the RNeasy Lipid Tissue kit (Qiagen, no. 74804, Hilden, Germany). RNA-Seq libraries were constructed from 100 ng RNA using the NEBNext Ultra II Directional Library Prep Kit for Illumina (New England Biolabs, no. E7760, Ipswich, USA) in combination with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, no. E7490, Ipswich, USA). The samples were combined at equimolar amounts and sent out for sequencing on an Illumina device in 150 bp paired-end mode (Genewiz, Leipzig, Germany). A total of 1.470 million reads, corresponding to an average of 19.5 million reads per sample, were obtained.

Differential expression analysis

RNA-Seq reads were further analyzed using the OmicsBox bioinformatics platform (v. 2.0.36,

www.biobam.com). Unless indicated otherwise, all tools used for differential expression analyses are accessible within the OmicsBox platform. RNA-Seq reads were preprocessed by Trimmomatic (Bolger et al. 2014) to remove sequencing adapters, low-quality sequences, and short reads from the dataset. The quality-trimmed reads were mapped to the *B. terrestris* genome assembly (Bter_1.0, GCA_000214255.1, downloaded from metazoa.ensembl.org) using STAR (Dobin et al. 2013). A gene-specific count table was created from the mapping files using HTseq (Anders et al. 2015) and differentially expressed genes were identified by edgeR (Robinson et al 2010), respectively. Functional annotation of the*B. terrestris* genome was based on annotation release v. 102 (available in gff3 format from metazoa.ensembl.org). Since 4,975 of the 12,008 genes did not contain any functional annotation, the functional annotation workflow of the OmicsBox platform was used to update the published annotation with additional information. In brief, the coding sequences of unannotated genes were used to extract functional annotations from refseq_protein database (www.ncbi.nlm.nih.gov) and InterProScan (www.ebi.ac.uk). These we then fed into the GO mapping and annotation tools of the pipeline and finally merged to the existing functional annotations. Gene Set Enrichment Analyses (GSEA; Subramanian et al. 2005) were performed using ranked list of genes (rank = sign(logFC) * -log10(p-value); FC: fold-change) and gene sets defined by Gene Ontology's functional annotations. For the functional network analysis of enriched GO terms we used ClueGo (v. 2.5.9; Bindea et al. 2009) and CluePedia (v. 1.5.9; Bindea et al. 2013) plugins in Cytoscape (v. 3.9.1; Shannon et al. 2003). We used the packages *ggplot2* (Wickham 2016), *ggpubr* (Kassambra 2020) and *pheatmap* (Kolde 2019) to plot transcriptome data in R 4.2.1 (R Core Team 2022).

Results

Effect of pollutants on the bumblebee gut microbiome

Amplicon sequencing of the bacterial 16S rDNA fragments yielded a total of 2,425,928 raw reads. After quality filtering and removal of unassigned sequences, we also removed samples with a sampling depth below 3900 reads (n=7), all from DEP treatment, to ensure adequate sampling depth (13 DEP replicate samples remained in the analysis). In the remaining samples we obtained 1,856,025 16S rDNA gene sequences with a mean of 25,425 reads per sample (n=73), corresponding to 468 amplicon sequence variants (ASVs). Sample-based rarefaction curves suggest a sufficient sequencing depth for a representative coverage of the microbiome as most of the samples reach a plateau (**Figure A1**).

Taxa abundance

On the genus level, the most common bacterial taxa (> 1 % in at least one treatment) were: *Gilliamella, Snodgrassella, Lactobacillus, Asaia, Bombiscardovia, Methylorubrum* and*Bombilactobacillus.* The relative abundance of the most common genera for each sample shows a different microbial composition in the DEP treatment compared to the other treatment groups (**Figure 1**).

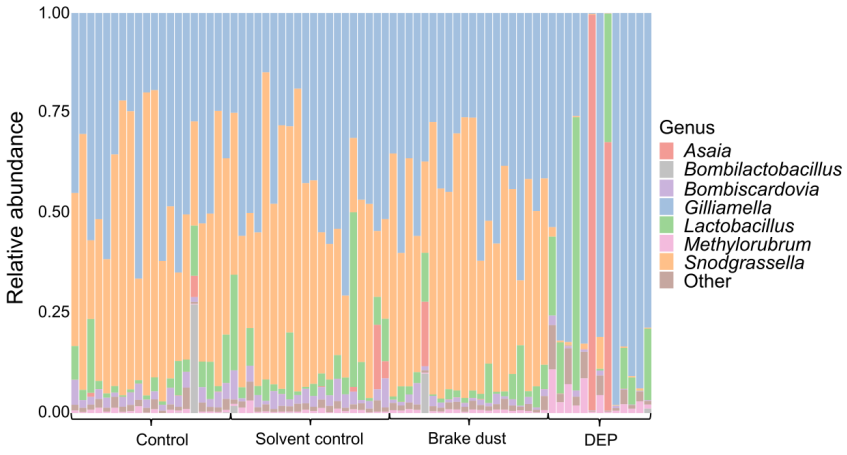

Figure 1: Relative abundance of the most common bacterial genera for each sample. Samples are arranged according to treatment.

While the relative abundance of ASVs did not differ between control, solvent control, and brake dust, DEP treatment had 16 differentially abundant ASVs compared to the control, according to DESeq2 (**Figure 2** , **Table A1**). Eleven ASVs had a higher abundance in the DEP treatment than control. Five ASVs had reduced abundance in comparison to the control treatment. A more conservative approach to identify differential abundance is ALDEx2, which revealed five ASVs with significantly altered abundance in the DEP treatment compared to the control: *Snodgrassella* 1 + 2, Neisseriacae,*Lactobacillus bombicola* , and *Bombiscardovia* (**Table A2**).

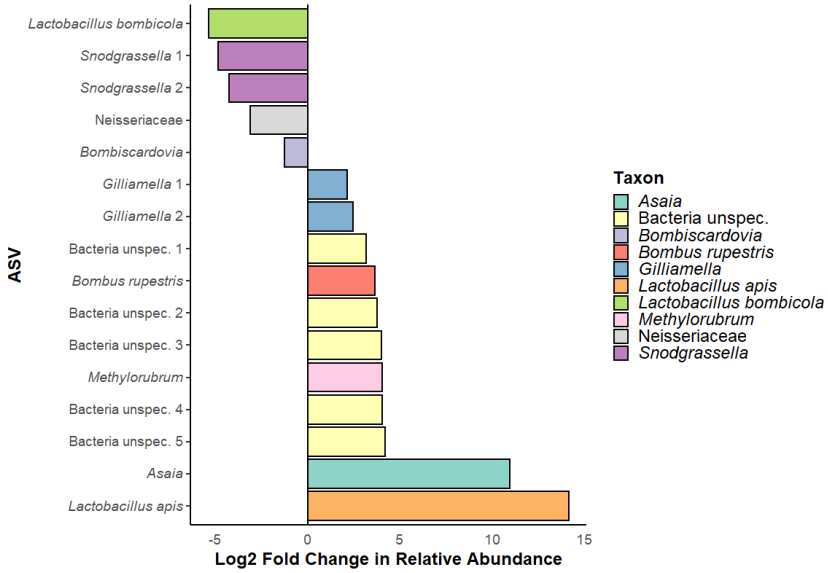

Figure 2: Log2 fold change in relative abundance of ASVs in the DEP treatment in comparison to the control. Cutoff for inclusion of ASVs in this plot was FDR ($=P_{adj}$) < 0.01. Colors represent most specific taxonomic label.

?-diversity of the gut microbiome

The number of observed ASVs did not differ between treatments (GLM with gamma distribution: $F_{3,69} = 0.3008$, $P = 0.825$;**Figure 3A**). Pielou's evenness differed between treatments (Kruskal-Wallis rank sum test: $X^2 = 23.296$, df = 3, P < 0.001; **Figure 3B**). The DEP treatment had a significantly lower evenness than the other treatments (Dunn's comparisons with Benjamini-Hochberg (BH) adjusted p-values: DEP vs. control $P < 0.001$, DEP vs. solvent control $P < 0.001$, DEP vs. brake dust $P < 0.001$; Figure**3B**). Shannon diversity differed between treatments (Kruskal-Wallis rank sum test: $X^2 = 14.642$, df = 3, P = 0.002; **Figure 3C**). The DEP treatment had a significantly lower diversity than the other treatments (Dunn's comparisons with BH adjusted p-values: DEP vs. control $P = 0.003$, DEP vs. solvent control $P = 0.001$, DEP vs. brake dust $P = 0.010$; **Figure 3C**). Faith's PD differed between treatments (Kruskal-Wallis rank sum test: $X^2 = 10.777$, df = 3, P = 0.013; **Figure 3D**). Faith's PD in the DEP treatment was significantly higher than in the other treatments (Dunn's comparisons with BH adjusted p-values: DEP vs. control $P = 0.009$, DEP vs. solvent control $P = 0.009$, DEP vs. brake dust $P = 0.007$;**Figure 3D**).

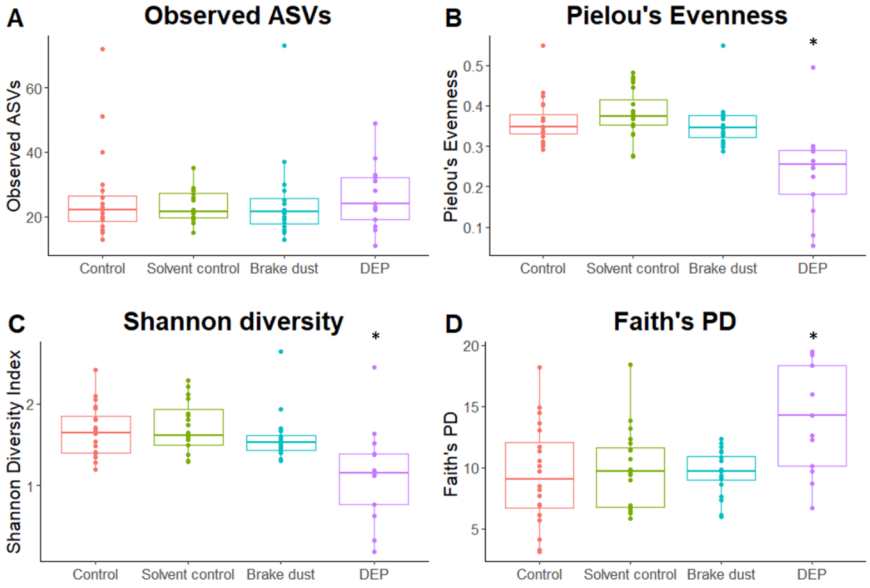

Figure 3: ?-diversity of the bumblebee gut microbiomes for the different treatments. A) Observed ASVs, B) Pielou's Evenness, C) Shannon Diversity, D) Faith's PD. Asterisks indicate significant differences compared to the other treatments (P < 0.05). Boxplots show median, first, and third quartile. Dots represent individual data points.

?-diversity of the gut microbiome

The community composition of the bumblebee gut microbiome differed between treatments indicated by significant differences between the robust Aitchison distances (Overall PERMANOVA pseudo-$F_{4, 73} = 16.844$, $P = 0.001$). Microbial community composition of the DEP treatment differed from all other treatments (Pairwise-PERMANOVA with BH adjusted p-values; DEP vs. control: pseudo-F = 32.247, $P = 0.002$; DEP vs. solvent control: pseudo-F = 30.651, $P = 0.002$; DEP vs. brake dust: pseudo-F = 25.699, $P = 0.002$). We found no differences between the other treatments (Pairwise-PERMANOVA with BH adjusted p-values: $P > 0.05$) (**Figure 4**).

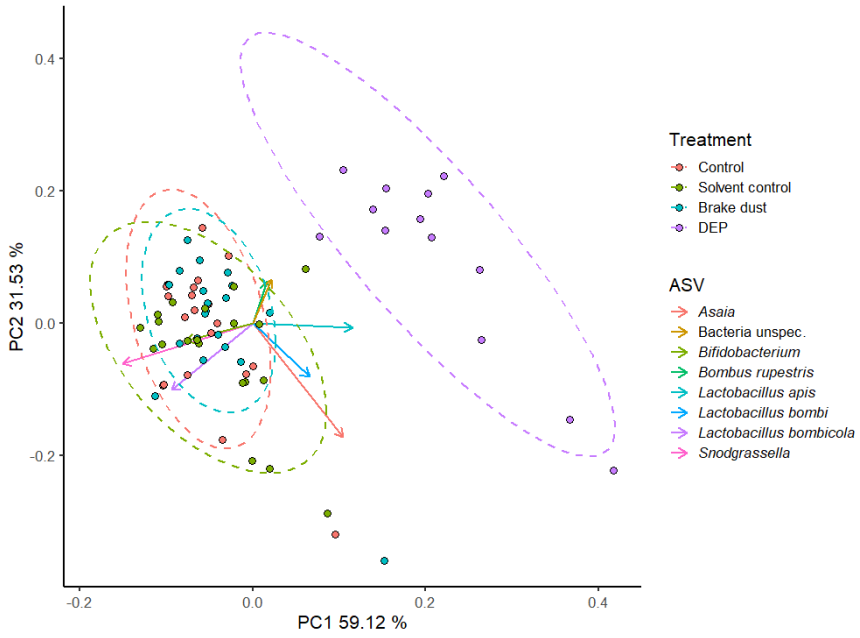

Figure 4: DEICODE distances based on Robust Aitchison Principal Components Analysis. Points represent single samples colored according to treatment. Arrows represent Euclidian distances from the origin and indicate ASVs with strong influence on the principal component axis. Ellipses show 95% confidence interval for multivariate t-distribution of each treatment. The ASV of the eukaryotic organism Bombus rupestris can explained by a remaining non-specificity of the used primers (as analyzed by TestPrime, www.arb-silva.de).

Effect of pollutants on bumblebee gene expression

In the transcriptome analysis, we focused only on biologically relevant comparisons of treatments to prevent unnecessary inflation of reported results. We compared control vs. solvent control, control vs. DEP, control vs. brake dust, and flight control vs. DEP flight. The analysis for differently expresses genes (DEGs) revealed differences between our treatments. In total, 324 genes were differentially expressed in the DEP treatment compared to the control (low-count gene filter settings: CPM Filter=1, samples reaching CPM Filter=2). 165 genes were upregulated (LogFC $>$ 1) and 159 genes downregulated (LogFC $<$ -1), respectively (**Figure A2**). In the brake dust treatment only one gene was differentially expressed (upregulated) in comparison to the control. In the solvent control, there were no differentially expressed genes compared to the control. In the DEP flight treatment, we found no differentially expressed genes in comparison to the flight control.

The variation in gene expression of bumblebee workers is clearly distinct between the control and the diesel exhaust particle treatment (**Figure 5**). The clear separation between the treatments across all samples indicates substantial differences in gene expression of bumblebees when exposed to DEP orally. The reliability of this difference in gene expression is confirmed by a cluster analysis which shows a definite clustering by treatment rather than by colony (**Figure 6**). The other treatments are not clearly distinct in a nMDS plot and indicate no differences in gene expression (**Figures A3-A5**), thus we do not conduct further analyses on these comparisons.

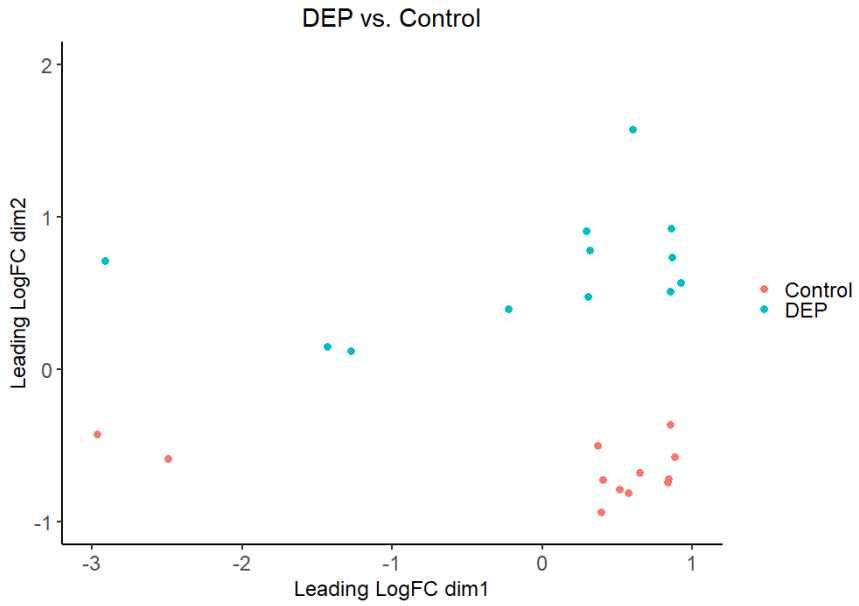

Figure 5: Non-metric multidimensional scaling plot based on the log2 fold changes (FC) between control and DEP treatment. The axes of the nMDS plot represent dimensional reductions of gene expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to treatment.

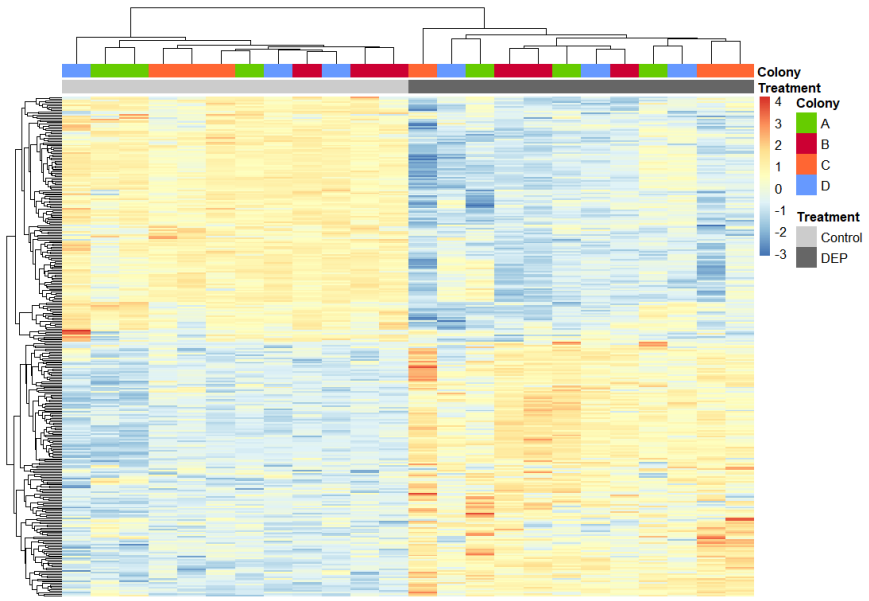

Figure 6: Heatmap showing hierarchical clustering of samples (x-axis) and differentially expressed genes (y-axis) for the control and DEP treatment. Cluster color represents the expression level of genes in $\log_2$CPM (Counts per million reads).

The 324 differentially expressed genes in the DEP treatment were annotated to gene ontology (GO) terms, which describe gene properties and group each into one of three categories: Cellular component, molecular

function, and biological process. We used GO enrichment analysis to find the most over- and underrepresented term. The 30 most significantly upregulated GO terms in the DEP treatment include protein-binding functions, enzyme complexes and metabolic, especially catabolic, processes (**Figure 7A**). The 30 most significantly downregulated GO terms in the DEP treatment include transferase activity, mitochondrial and organelle membranes, as well as metabolic, especially biosynthetic, processes (**Figure 7B**).

The functional network analysis based on ?-Score [?] 0.4 for differentially expressed GO terms with FDR [?] 0.05 in the DEP treatment shows clustering to specific functional groups (**Figure A6A**). Upregulated functions are related to phosphorylation, regulation of metabolic process, guanyl nucleotide binding, and signal transduction (**Figure A6B**). Downregulated functions are related to mitochondria, lipid metabolic processes, the endoplasmic reticulum, and phospholipid biosynthetic processes (**Figure A6C**).

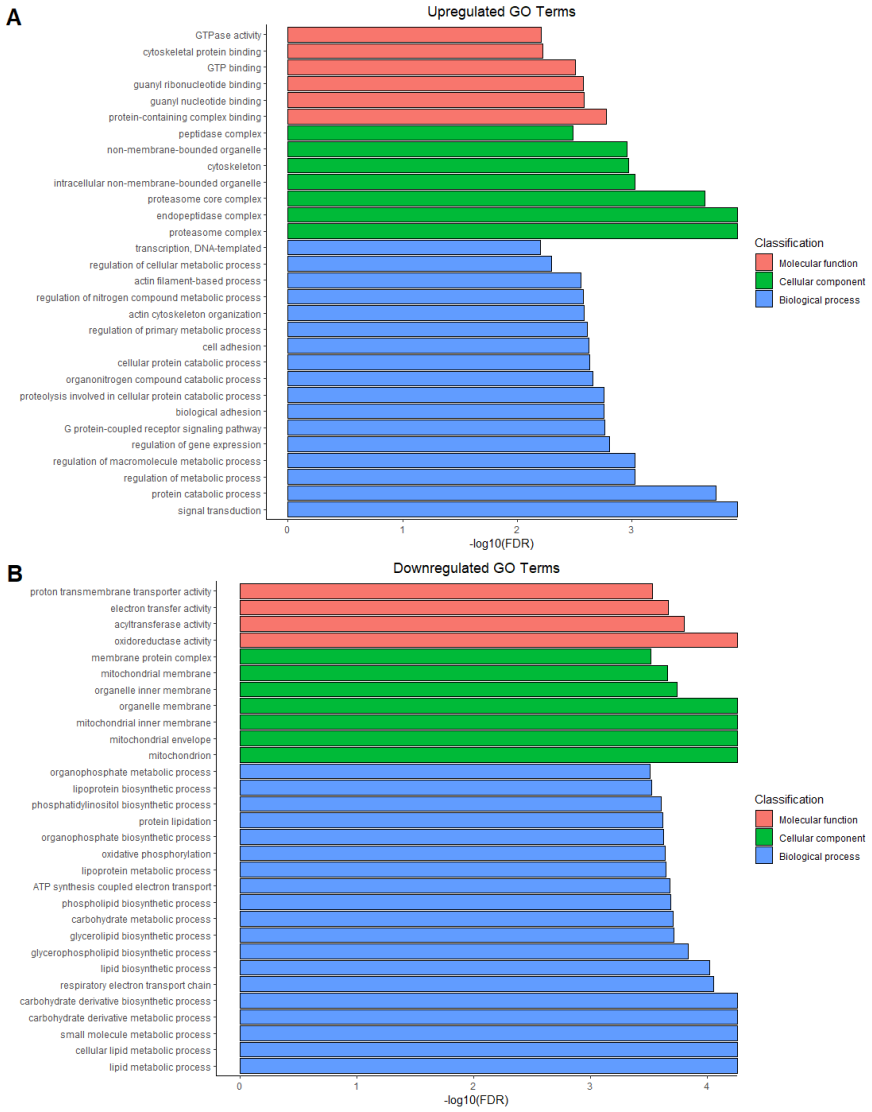

Figure 7: Gene ontology terms of A) the 30 most significantly upregulated and B) downregulated genes in the DEP treatment colored by category and sorted by -log10FDR.

Discussion

In this study, we found that oral exposure to diesel exhaust particles (DEPs) changes the gut microbiome and gene expression of bumblebee workers, while DEP exposure via air did not. Brake dust, the second pollutant we tested via oral exposure, did not induce changes in the gut microbiome or gene expression in the bumblebee workers.

While the composition of the microbial gut community in control, solvent control, and brake dust exposure treatment was similar, we detected major shifts in the DEP treatment. This raises several interesting questions: 1) How do DEPs affect the bacteria to induce changes in the gut microbiome composition? 2) Which components in diesel exhaust are responsible for the observed changes? Our hypothesis is that PAHs could be the component of DEP affecting bacteria directly. DEPs contain different PAHs, a class of organic compounds well-known to be toxic, mutagenic, and genotoxic to various life forms (Patel et al. 2020, Sun et al. 2021). Also, shifts in the microbial gut community due to PAH exposure have been reported in different animals, such as fish, sea cucumbers, or potworms (Enchytraeidae) (DeBofsky et al. 2020, DeBofsky 2021, Ding et al. 2020, Quintanilla-Mena et al. 2021, Zhao et al. 2019). Therefore, we suspect PAHs to be the leading cause of changes in the bumblebee gut microbiome in our study. However, the large amount of elemental carbon in DEPs, may itself provide another explanation. The DEPs may function like activated carbon with its large surface-area-to-volume ratio and may adsorb microbes that are then discharged by excretion (Naka et al. 2001, Rivera-Utrilla et al. 2001, Wichmann 2007). Even though activated carbon has no direct negative impact, constant adsorption and discharge might disrupt the bacterial community resulting in the compositional and quantitative changes similar to those observed in our study.

The bacterium *Snodgrassella* , one of the dominant core bacteria in undisturbed gut microbiomes of bumblebees (Hammer et al. 2021), is nearly absent after the DEP exposure. *Snodgrassella* , together with *Gilliamella* , forms a biofilm coating the inner wall of the ileum (Hammer et al. 2021, Martinson et al. 2012). Both, host and symbionts could profit from this biofilm formation as it prevents bacteria from washout and enables the formation of a syntrophic network (Kwong et al. 2014, Powell et al. 2016, Zhang et al. 2022). Additionally, the biofilm could protect the host against gut parasites, such as *C. bombi,* who need to attach to the gut wall to persist (Koch et al. 2019, Näpflin & Schmid-Hempel 2018). However, the mutualistic relationship between the microbes seems to be disrupted by DEP exposition, as *Snodgrassella* abundance is extremely diminished. In contrast, *Gilliamella* increases in relative abundance after DEP exposure. This indicates that *Gilliamella* may be able to form a biofilm independently from *Snodgrassella* . A relatively simple explanation for the higher relative abundance of*Gilliamella* might be that the reduction of *Snodgrassella*leaves *Gilliamella* as the only dominant bacterium in the gut and therefore *Gilliamella* might thrive better or fill the void.*Snodgrassella* seems especially prone to pollutants, as Rothman et al. (2020) already reported a decrease in its relative abundance after exposure of bees to copper, selenate, or glyphosate. Additionally, we found an unknown bacterium from the family Neisseriaceae, the same family to which also *Snodgrassella* belongs, having a lower relative abundance after DEP exposure. If this is a consistent result, it might indicate a general susceptibility of this family to DEPs.

The higher abundance of *Asaia* in the DEP treatment was driven by two samples, in which *Asaia* dominates the bacterial community with relative abundances of 99 % and 67 %, respectively. *Asaia*is a flower-associated acetic acid bacterium, which is commonly found in the gut of members of different insect orders, such as Hemiptera, Diptera, and Hymenoptera (Bassene et al. 2020, Crotti et al. 2009, Kautz et al. 2013). It can dominate the gut microbiome of *Anopheles*mosquitos, which is why it is considered a potential tool in malaria control (Capone et al. 2013, Favia et al. 2008). While there have been reports of *Asaia* in bumblebees, the dominance of *Asaia* in some of the DEP samples is rather uncommon (Bosmans 2018). DEPs might disrupt the natural microbiome community opening the door for opportunistic bacteria such as *Asaia* (Favia et al. 2007). Even though we kept the bumblebees in this experiment indoors throughout their lives, *Asaia* bacteria may derive from pollen fed to the bumblebees before the start of the experiment.

We detected an interesting pattern in the genus *Lactobacillus* , one of the core gut bacteria of bumblebees (Hammer et al. 2021). While the species *L. bombicola* , a bumblebee-associated bacterium, has a lower abundance after DEP exposure, the abundance of the honeybee-associated *L. apis* increases. Again, the

disruption of the original microbiome caused by DEPs might explain that foreign bacteria can establish themselves in the microbiome. As the pollen fed to the bumblebees before the experiment was collected by honeybees, it could be the source of *L. apis* .

The DEP-induced changes in the gut microbiome may affect bumblebee health, as core bacteria could prevent infections by parasites. The abundance of *Gilliamella* , *Lactobacillus* and*Snodgrassella* is negatively correlated with the parasites*Crithidia* and *Nosema* , while non-core bacteria are more abundant in infected bumblebees (Cariveau et al. 2014, Koch et al. 2012, Koch & Schmid-Hempel 2012, Mockler et al. 2018). The biofilm formation of *Snodgrassella* and *Gilliamella* may form a physical barrier to the trypanosome *C. bombi* which needs to attach to the ileum wall to persist (Koch et al. 2019, Näpflin & Schmid-Hempel 2018). The disruption of this biofilm and the higher abundance of non-core bacteria, such as *Asaia* , may increase the parasite susceptibility of bumblebees exposed to DEPs.

The transcriptome analysis revealed significant changes in gene expression after oral exposure of bumblebees to a sublethal dose of DEPs. In total, 165 genes were upregulated, and 159 genes were downregulated. GO enrichment analysis and network analysis indicate that these changes could be related to a general stress response against pollutants. While upregulated GO terms involve many metabolic and catabolic processes, downregulated GO terms include metabolic and biosynthetic processes. DEP exposure might deplete stored reserves causing the observed changes as a consequence of higher energetic costs. Changes in metabolism seem to be a typical reaction to pollutants in insects which seems reasonable as they often interfere with biochemical processes. Transcriptional changes in bumblebees and honeybees exposed to sublethal doses of neonicotinoids are mainly linked to metabolic processes (Bebane 2019, Colgan 2019, Gao et al. 2020, Shi et al. 2017). Exposure to heavy metals or PAHs induces similar changes in spiders, mosquitos, moths, and fireflies (Chen et al. 2021, David et al. 2010, Li et al. 2016, Zhang et al. 2019, Zhang et al. 2020). Even though the changes differ in detail, certain processes seem commonly involved in the response to pollutants. Consistent with our findings, exposure to insecticides or PAHs affects mitochondrial functioning, an important part of the insect energy metabolism (Colgan et al. 2019, Zhang et al. 2019, Zhang et al. 2020). This supports the idea of increased energy demand caused by pollutants (Beyers et al. 1999, Calow 1991). We also observed an upregulation of signal transduction in our study, similar to observations in honeybees and fireflies exposed to Imidacloprid and the PAH benzo(a)pyrene, respectively (Gao et al. 2020, Zhang et al. 2019, 2020). Typically, chemical stressors, such as PAHs, insecticides, and heavy metals, affect genes associated with detoxification processes and drug metabolism (Chen et al. 2021, David et al. 2010, Gizaw et al. 2020, Zhang et al. 2019). However, in our study, we did not find any differentially expressed detoxification-related genes. Possibly the number of PAHs attached to the DEPs was not enough to trigger a reaction that would lead to a measurable increase in detoxification. Overall, the observed changes in gene expression after oral DEP exposure of bumblebees resemble a general stress response to pollutants.

In contrast to oral exposure, we did not find any effect on gene expression after exposure of bumblebees to DEPs via the air. To cause changes, DEPs need to enter the tracheal system or attach to sensory organs, such as the antennae. The exposure of bumblebees for three minutes per day may not have been enough to affect them. Particles on the antennae may have been removed quickly by cleaning behavior and the spiracles seem to be an effective protective barrier against the uptake of particles into the tracheae (Harrison 2009, Schönitzer 1986). Thus, our results should be taken with care because probably only very few particles entered the tracheal system of the bumblebees.

Unlike DEPs, oral exposure to brake dust particles did not affect the gut microbial community nor the gene expression of the bumblebees. However, some concerns remain about the experimental procedure. For one, we did not use brake dust from a real braking scenario, but rather artificially milled brake pads. Dust derived from them may have different physicochemical properties. Milled brake dust particles have a much higher mean particle size than DEPs (10 μm vs. 0.01 μm). As we defined treatment concentration per weight, these different physical properties lead to big differences in the particle counts of the treatment solutions, i.e. solutions with brake dust contained far fewer particles than those with DEPs. Moreover, large brake dust particles tend to sink to the bottom of the feeding syringes which might have reduced the particle uptake.

While brake dust seems not to affect the bumblebees, further studies are needed to address the indicated limitations of the present study.

Taken together, the results from our microbiome and transcriptome analysis indicate potential consequences for insect health, here shown in bumblebees, after oral DEP exposure. Gut dysbiosis may increase the susceptibility of bumblebees to pathogens, while a general stress response may lower available energetic resources. To evaluate these hypotheses further studies should investigate the combined effect of DEP exposure and other stressors, such as parasites, limited food availability, or abiotic factors. Bumblebees may be able to compensate for facing one stressor but will eventually be overstrained by multiple stressors. Additionally, whole colony experiments would add to the evaluation of DEPs as a potential contributor to insect losses, as effects may be small on the individual level but accumulate on the colony level.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The microbiome and RNA-Seq sequencing data were deposited at NCBI's Sequence Read Archive (SRA) under Bioproject numbers PRJNA907197 (16S microbiome sequencing) and PRJNA907822 (transcriptome sequencing), respectively.

Author contributions

DS, AW, OO, and HF conceived the idea, designed the experiment, and wrote the manuscript. AM, TH, TO, NL, and DB produced and analyzed the particulate matter. DS, MR, and AW carried out the experiment. DS and AW performed the data analysis. DS, AW, OO, and HF interpreted the results. All authors read and approved of the final manuscript.

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Appendix

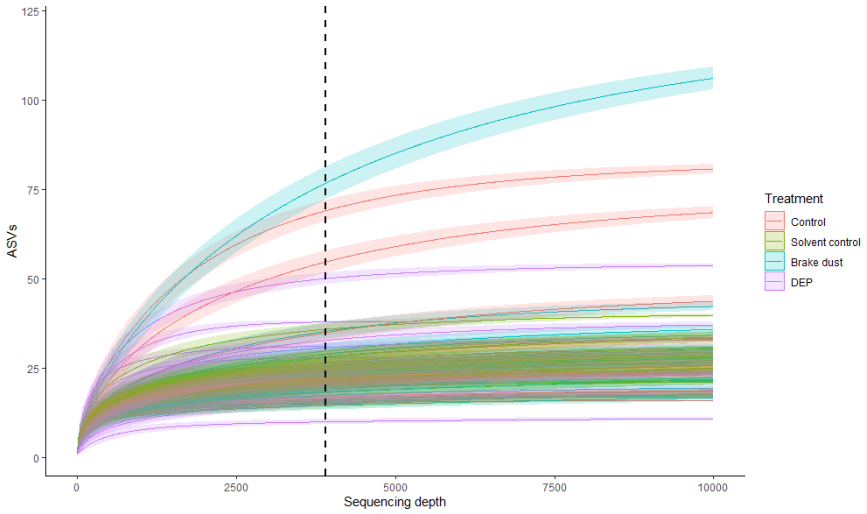


Figure A1: Rarefaction curve of each sample, colored according to their respective treatment. X-Axis is cut off at 10000 reads. Vertical dashed line indicates sequencing depth of 3900.

Table A1: Differentially abundant ASVs comparing DEP to the control treatment, according to DESeq2 (cutoff: FDR < 0.01). Positive Log2 fold changes indicate higher abundance in the DEP treatment.

ASV	Log2 Fold Change	P_{adj} (= FDR)	Feature ID
Lactobacillus bombicola	-5.372	<0.001	ac3366c90455cdc1a4ad414f21215a91
Snodgrassella 1	-4.848	<0.001	f9dff838e1ab76a58a54df65a2457d5a
Snodgrassella 2	-4.256	<0.001	8f7166172175c35bbfc8fa4dc5ef58b8
Neisseriaceae	-3.108	<0.001	f1ae3848b7e710b5da56f2a447ae0234
Bombiscardovia	-1.251	0.010	bf7591505d4138d52e3a9c537c958fa1
Gilliamella 1	2.146	<0.001	36aed5b1dc9b5c1a2844e58f2d34b1f5
Gilliamella 2	2.473	<0.001	1e232cdf347e2b62b3b1d7347e891797
Bacteria unspec. 1	3.162	0.001	6445d5095ad81f1b73aa974a171ebce6
Bombus rupestris	3.645	<0.001	6d53feb4ee4fac60aba11969e1e5fc01
Bacteria unspec. 2	3.768	0.004	101de948d3a66ac329a31fd5f92c00d5

ASV	Log2 Fold Change	P_{adj} (= FDR)	Feature ID
Bacteria unspec. 3	4.008	<0.001	7ebb40e08aa315a3ab9ae5fb0b47ae34
Methylorubrum	4.025	<0.001	92f1720367db58c68a96eceb9feb416a
Bacteria unspec. 4	4.030	<0.001	5c70c440562c05d292daf0c5b4694ef4
Bacteria unspec. 5	4.201	<0.001	a6ddcd6498df4ed3d6c3e05663f658fb
Asaia sp.	10.960	<0.001	49d46d00a93443b060707ab2db8ba82d
Lactobacillus apis	14.158	<0.001	96d14363f547715b65bf7d8ad1d31d17

Table A2: Differentially abundant ASVs comparing DEP to the control treatment, according to ALDEx2. Negative effect indicates higher abundance in the control. P_{adj} = Expected Benjamini-Hochberg corrected P value of Wilcoxon test. Effect = median effect size (diff.btw/max(diff.win)).

ASV	Effect	P_{adj}	Feature ID
Snodgrassella 2	-5.516	<0.001	8f7166172175c35bbfc8fa4dc5ef58b8
Neisseriaceae	-2.659	<0.001	f1ae3848b7e710b5da56f2a447ae0234
Lactobacillus bombicola	-2.393	<0.001	ac3366c90455cdc1a4ad414f21215a91
Snodgrassella 1	-2.356	<0.001	f9dff838e1ab76a58a54df65a2457d5a
Bombiscardovia	-2.092	<0.001	bf7591505d4138d52e3a9c537c958fa1

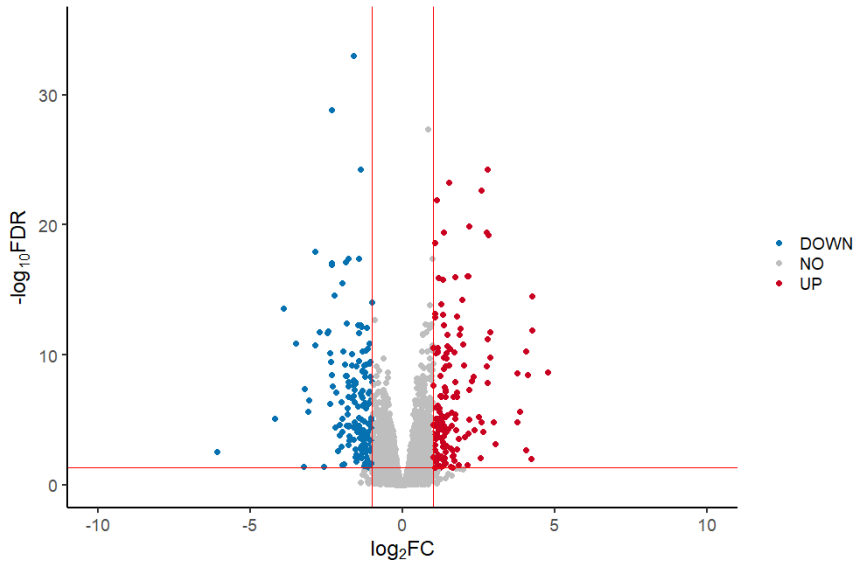


Figure A2: Differential expression of genes in the DEP treatment in comparison to the control. Blue dots represent significantly downregulated genes, red dots represent significantly upregulated genes. The horizontal red line marks a $-\log_{10}$(FDR=0.05). The two vertical red lines mark a $\log_2$FC of -1 and 1, respectively.

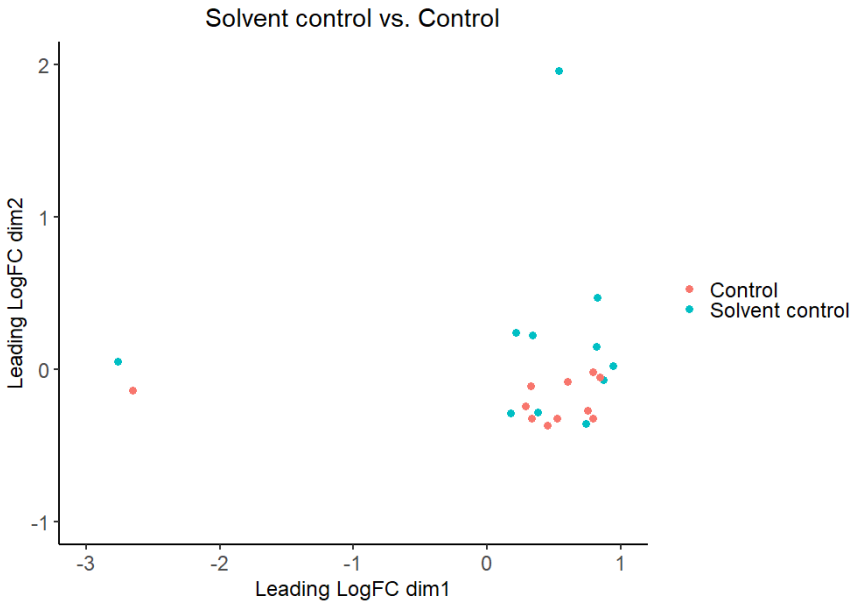


Figure A3: Non-metric multidimensional scaling plot based on the log2 fold changes (FC) between control and solvent control. The axes of the nMDS plot represent dimensional reductions of genes expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to the respective treatment.

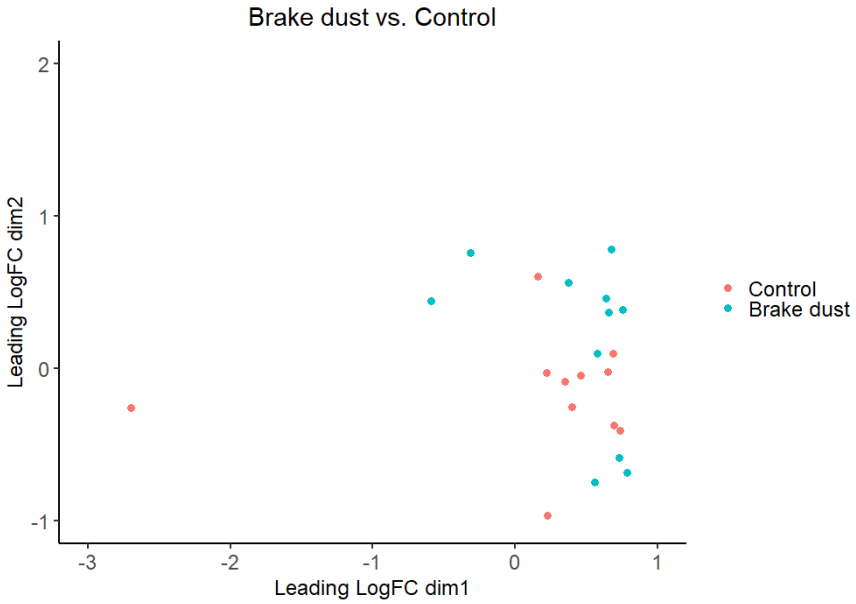


Figure A4: Non-metric multidimensional scaling plot based on the log2 fold changes (FC) between control and brake dust treatment. The axes of the nMDS plot represent dimensional reductions of genes expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to the respective treatment.

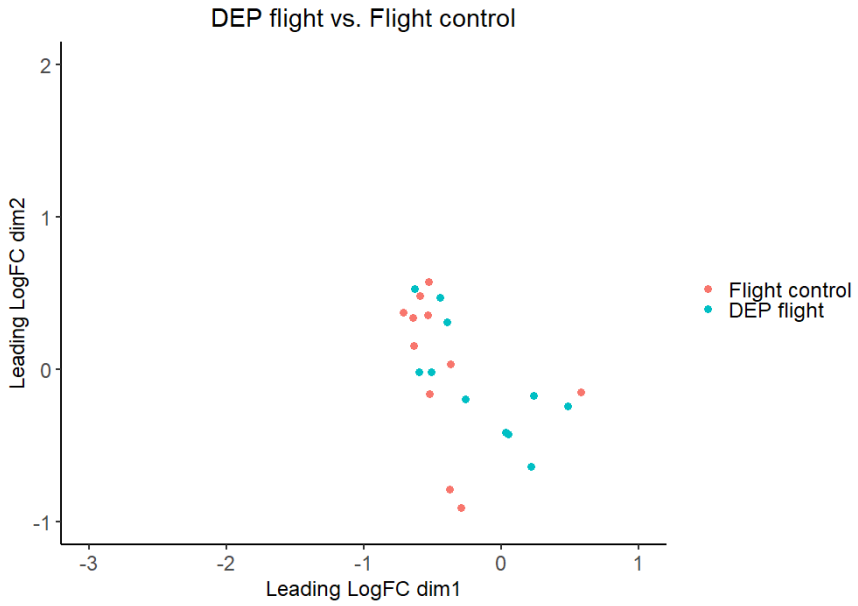


Figure A5: Non-metric multidimensional scaling plot based on the log2 fold changes (FC) between flight control and DEP flight treatment. The axes of the nMDS plot represent dimensional reductions of genes expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to the respective treatment.

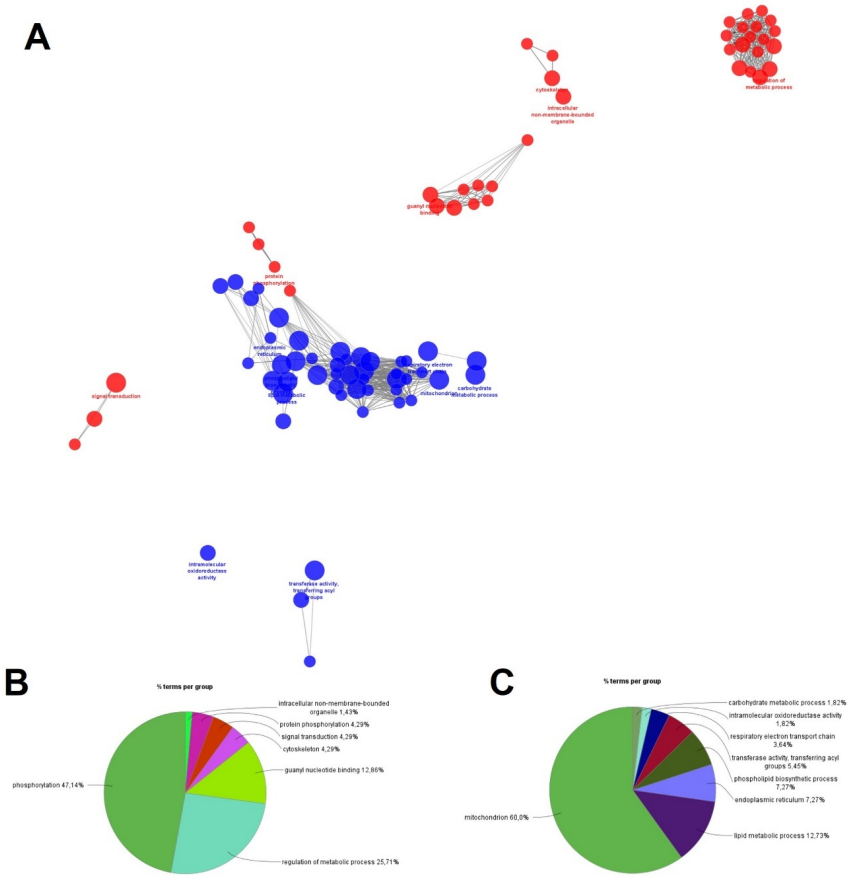
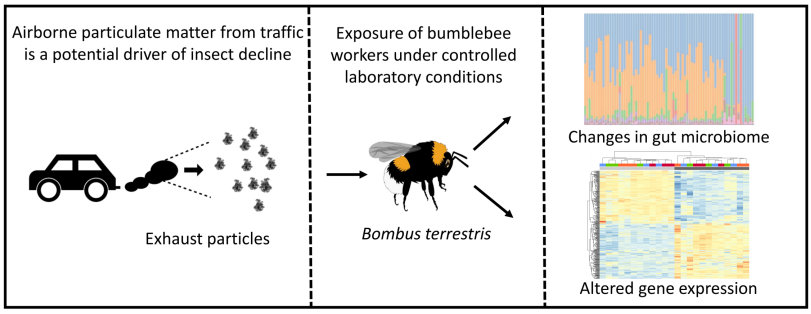


Figure A6: Network analysis of enriched gene terms and functional groups in the DEP treatment based on Kappa-Score [?] 0.4 for GOs with FDR [?] 0.05 using the ClueGo and CluePedia plugins of Cytoscape. (A) Functionally grouped network of upregulated (red) and downregulated (blue) gene ontologies. (B) pie chart with functional groups, including specific terms upregulated in the DEP treatment. (C) pie chart with functional groups, including specific terms downregulated in the DEP treatment. The area covered by each group represents the relative number of GO terms within each group. The most significant term each group is labelled.



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