# Structural and metabolic profiling of Lycopersicon esculentum rhizosphere microbiota artificially exposed at commonly used Non-Steroidal Anti-Inflammatory Drugs

**Emoke Dalma Kovacs** ( dalmaemokekovacs@gmail.com )
ICIA, INCDO-INOE 2000
**Szilaghi-Dumitrescu Luminita**
UBB: Universitatea Babes-Bolyai
**Tian Di**
Capital Normal University

**Research Article**

**Keywords:** rhizosphere microbiota, PLFA, metabolic activity, volatile organic compounds

**Posted Date:** July 26th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-740188/v1



**Version of Record:** A version of this preprint was published at Microorganisms on January 24th, 2022.
See the published version at https://doi.org/10.3390/microorganisms10020254.

# Abstract

In this study the effect of common non-steroidal anti-inflammatory drugs on *Lycopersicon esculentum* rhizosphere microbiota was monitored. Results evidenced that rhizosphere microbiota abundance decreased especially under exposure to diclofenac and ibuprofen while fungal/bacteria ratio changed significantly with exposure to diclofenac and ketoprofen. Compared with control samples the average amount of ratio of gram negative/gram positive bacteria was higher in rhizosphere soil contaminated with ibuprofen and lower in case of diclofenac contamination. Carbon source consumption increased with time of assay in case of control samples and those contaminated with diclofenac. This suggests that rhizosphere microbiota under contamination with diclofenac consume higher amount of carbon although do not consume a larger variety of its sources. In case of contamination with ibuprofen and ketoprofen the consumption of carbon source presents a decreasing tendency after day 30 of the assay. Rhizosphere microbiota emitted volatile organic compounds were also monitored. Volatile compounds belonging to alcohol, aromatic compounds, ketone, terpene, organic acids, aldehyde, sulphur compounds, esters, alkane, nitrogen compounds, alkene and furans were detected in rhizosphere soil samples. Among these, terpene, ketone, alcohol, aromatic compounds, organic acids, and alkane were the most abundant compound classes, but their percentage changed with exposure at diclofenac, ketoprofen and ibuprofen. Such changes in abundance, structure, and metabolic activity of *Lycopersicon esculentum* rhizosphere microbiota under exposure to common non-steroidal anti-inflammatory drugs make as to suppose that there is a probability to also change ecosystem services provided by rhizosphere microbiota.


# Introduction

Diclofenac, ibuprofen and ketoprofen are common non-steroidal anti-inflammatory drugs (NSAIDs) that are often reported in environmental assessment studies (Chaturvedi et al., 2021; Praveenkumarreddy et al., 2021; Fedaku et al., 2019; Madikizela and Chimuka, 2017;). This is because of their high consumption rate (Kamat et al., 2020; Ayukekbong et al., 2017) and improper removal during wastewater treatment processes (Alenzi et al., 2021; Courtier et al., 2019; Cycon et al., 2016; Garcia-Rodriquez et al., 2013; Sebok et al., 2008;). They reach soil system through reuse either of treated municipal wastewater in irrigation purposes (Mordechay et al., 2021; Grabicova et al., 2020;), or of the resulted sludge and biosolids as fertilizers (Mejias et al., 2021; Xu et al., 2009). Their common occurrence in soil environment raised high concern because of continuous input (Quintelas et al., 2020; Duan et al., 2013), subsequent accumulation potential (Mordechay et al., 2021;) and their potential ecotoxicological effects on nontargeted living organisms at different trophic levels (Mejias et al., 2021; Grabicova et al., 2020; Papaioannou et al., 2020; Cycon et al., 2016).

Soil microbiota are key actors in soil processes, contributing significantly to numerous ecosystem services provided by soil (Imade and Babalola, 2021; Gianinazzi et al., 2010). They are involved in processes of nutrients cycling and organic matter degradation (Gama-Rodrigues, 2011; Kang et al., 2007). Microorganisms are also able to synthesize volatile organic compounds as alcohols, terpenes, ketones, alkanes, etc. (Ajilogba and Babalola, 2019; Ramirez-Guizar et al., 2017). These are secondary metabolites

with multiple ecological role and mechanisms of action. Reports started to highlight those volatile organic compounds emitted by soil microbiota can act as signalling molecules assuring distance communication between various organisms (Brown et al., 2021), can induce inhibitory activity against fungal spore germination (Orlandini et al., 2014) those changing microbiota structure, and can modulate enzyme activity (Lopez et al., 2021). In this way they can directly influence the aboveground biodiversity and productivity (Hofmann et al., 2016; Rode, 1995). Through the chemicals that microorganism cycle or release they can stimulate or inhibit plant development (Kang et al., 2007). Rhizosphere, the interface between plant roots and soil is one of the most abundant and dynamic system inhabited by microorganism (Bhattacharyya and Lee, 2016; Sanon et al., 2009). Rhizosphere microbiomes differ from that of soil microbiome. Although it is well acknowledged that the microbiota of rhizosphere has positive effects on plant development and heath (Fincheira et al., 2021), there are less knowledge on their structure, abundance, and function under challenging conditions. Considering the frequency of NSAIDs presence in soil environment (Chaturvedi et al., Mejias et al., 2021; 2021; Xu et al., 2009), studies are essential to unravel the functions of rhizosphere microbiota under exposure to frequently detected NSAIDs.

A major goal in ecology is to assure a more stable agrosystems development that can face current challenges. This could be achieved through achievement of a deeper knowledge on rhizosphere ecology and identification of rhizobiome chemical and biological diagnostics and signatures for identified issues. Microbiota are characterized as small organisms, therefore compared to other organisms they present a high surface area-to-volume ratio throughout they assure a large contact interface that interact with surrounding environment, respectively with surrounding contaminants (Pino-Otin et al., 2017). Under exposure to pharmaceuticals, soil microbiome biological parameters as structure, abundance and metabolic activity could suffer changes. Wang et al., (2019) assessed the toxic effects of enrofloxacin on soil enzymatic activities and showed that the activities of sucrase was inhibited significantly at all incubation periods. In studies on microbial utilization in the Biolog plates, they reported that utilization of metabolites was inhibited severely and reached essentially zero on 21 days, although they gradually decreased with the increasing of time (until day 21 of assay). In studies reported by Liu et al., (2012) it was found that soil microbial functional diversity and the capacity of soil microbial communities to utilize substrates were sensitive to sulfamethoxazole and chlortetracycline. Similarly, in triclosan ecotoxicity assessment of soil microbiota, Ramires et al., (2021) states that this antimicrobial agent inhibited the consumption pattern of carboxylic acids. In the study of influence of tetracycline presence from cow manure on soil microbiota, Chessa et al., (2016) evidenced that tetracycline only transiently influenced the microbiota abundance and functions.

As once exposed to different pharmaceuticals, soil microbiome could be changed in structure, abundance, or metabolic activity this in turn could have as consequence changes in key ecological processes of soil. After best of our knowledge, at present there are minor information on how presence of NSAIDs could shape rhizosphere microbiome structure, abundance, and metabolic activity. Moreover, there are no information on how presence of certain NSAIDs influence or not the secondary metabolites profile produced by rhizosphere microbiome. The main aim of this study was to identify if presence of

common NSAIDs as ibuprofen, diclofenac and ketoprofen could shape *Lycopersicon esculentum* rhizosphere microbiota (i.) abundance and phenotypic structure; (2) metabolic activity; and (iii.) secondary metabolites profile.

## Materials And Methods

# Experimental set-up:

Pharmaceuticals as ibuprofen ($C_{13}H_{19}O_2$), ketoprofen ($C_{16}H_{14}O_3$) and diclofenac ($C_{14}H_{11}Cl_2NO_2$), belonging to the class of nonsteroidal anti-inflammatory drugs, were selected for our study. Selection of these NSAIDs was based on their increased consumption and widespread occurrence in environment.

Argic phaeozem soil samples (0–40 cm), collected in April 2020 from Cojocna, Cluj county was used in this study. Main physical chemical properties of soil samples are listed in Table 1.

Table 1
Average values (n = 12) of physical chemical properties of studied soil samples

| Soil property | Argic phaeozem |
|---|---|
| Clay | 27.2 |
| Sand | 16.1 |
| Silt | 56.7 |
| Texture | Silty Clay Loam |
| Moisture ($cm^{-3}·cm^{-3}$) | 0.344 |
| Soil temperature (°C) | 10.4 |
| Organic carbon (%) | 6.2 |
| pH | 5.9 |

All soil samples were tested to be free of studied NSAIDs according with method described elsewhere (Kovacs et al., 2021). Samples were artificially contaminated individually with each pharmaceutical in part (0.5 $mg·kg^{-1}$ ibuprofen, 0.2 $mg·kg^{-1}$ ketoprofen, 0.7 $mg·kg^{-1}$ diclofenac) as previously described (Kovacs et al., 2021). For each rhizosphere soil sampling period individual pots in triplicate were prepared for each soil type vs. pharmaceutical as presented in schematic diagram of experiment set-up (Fig. 1). 14 days old tomato seeds (*Lycopersicon esculentum*) were planted in contaminated soils pots prepared one day before and allowed for development in a laboratory climate chamber with the following day-night cycle conditions: day – 14 h of light, 25 °C; night – 10 h of darkness, 18 °C. The soil water content was adjusted to assure a 58 % water holding capacity (WHC) during the study.

# Rhizosphere microbiota analysis through PLFA approach:

Rhizosphere soil microbiota phenotypic structure and abundance assessment was done considering phospholipids derived fatty acids (PLFA) gas chromatographic analysis. Rhizosphere soils were sampled from pots contaminated with pharmaceuticals (Fig. 1) after very- short term exposure (day 1), short term exposure (day 7), mid term exposure (day 30) and long-term exposure (day 60).

Extraction of PLFA was performed on 1 g of freeze-dried (Labconco FreeZone 6 freeze-dry system, MO, USA) soil according with method described by Bligh and Dyer (1959) and modified by (Frostegard et al., 2011). Lipids were fractionated into phospholipids, glycolipids and neutral lipids using a silicic acid column (500 mg, Phenomenex, Torrance, CA). After mild alkaline methanolysis, 150 µL of extracts containing the fatty acids methyl esters was injected into a gas chromatograph with flame ionization detector (7890A GC-FID, Agilent Technologies, CA, USA). Fatty acids methyl esters separation was obtained using an 5% phenyl-methylpolysiloxane column (HP-Ultra 2, J&W Scientific, Folsom, CA, USA) with the following properties: 25 mm x 0.2 mm id., 0.33 µm film thickness. PLFAD1 method from the MIDI Sherlock™ Microbial Identification System (Microbial ID, Inc., Newark, DE, USA) was used for phospholipids derived fatty acids separation. Gas chromatograph operation parameters are listed in Table 2. For interpretation of phospholipids derived fatty acids data bacterial fatty acid standards and software from MIDI Sherlock™ Microbial Identification System (Microbial ID, Inc., Newark, DE, USA) was used. Saprotrophic fungi were identified using 18:2ω6c PLFA biomarker (Frostegard et al., 2011), and ectomycorrhizal fungi with 18:2ω9c PLFA biomarker (Joergensen and Wichern, 2008). PLFA biomarkers as 18:2ω6c and 18:3ω3 were used for nitrogen reducing bacteria identification (Veuger et al., 2013), and 17:1ω7c, 10Me16:0, 17:1ω6, 15:1, i17:1ω7c, cy18:0ω7.8, i15:1ω7c and i19:1ω7c markers were used for sulphur reducing bacteria identification (Moulineux et al., 2015; Frostegard and Baath, 1996).

Table 2
GC-FID operation parameters for phospholipids derived fatty acids analysis from rhizosphere soils

| Parameter | Conditions |
|---|---|
| Inlet temperature | 280 °C |
| Split mode | 40:1 |
| Oven temperature program | 170 °C, increase with 28 °C·min$^{-1}$ until 288 °C, followed by a new increase with 60 °C·min$^{-1}$ until 310 °C. This final temperature was maintained constant for 1.25 min |
| Flow | 1.2 mL·min$^{-1}$ |
| Detector temperature | 300 °C |

# Rhizosphere microbiota responses evaluation:

Rhizosphere microbiota response to ibuprofen, diclofenac and ketoprofen was evaluated considering community level physiological profile (CLPP) and emitted volatile organic compounds (VOCs). Sampling

of rhizosphere soil samples from contaminated pots was done according with schematic diagram presented in Fig. 1.

*Community level physiological profile (CLPP)*: Assessment of metabolic activity of rhizosphere soil microbiota after exposure to NSAIDs was done using Biolog EcoPlate™ containing 31 different carbon sources. At each established sampling period, 2 g of rhizosphere soil samples were used to extract microbiota with 10 mL PBS solution. Extraction was allowed for 30 min through continuous mechanical shaking (LaboShake, Gerhardt Analytical System, Germany) after that followed by 1 h of rest. The mixture of soil suspension and supernatant was subjected to sonication and centrifugation as described by Lindahl and Bakken (1995) until to obtain the final soil lixiviate. Soil particles from obtained lixiviates were removed through low-speed centrifugation (1000 rpm for 1 min, LMC-3000 centrifuge, GrantBio, Italy). From this final solution 150 µL was added to each well of EcoPlate and incubated under dark conditions at 25°C for 3 days (LabCompanion, CA, USA). The optical density (OD) of each well was measured at λ = 590 nm using an SpectraMax iD3 Microplate Reader (Molecular Devices, CA, USA) and SoftMax Pro7 software (Molecular Devices, CA, USA) just after inoculation and once a day during incubation period.

### Emitted VOCs

Rhizosphere soil emitted volatile organic compounds content was determined through headspace-solid phase microextraction sampling using 85 µm polyacrylate fibre (Supelco Inc., Bellefonte, PA, USA). 1 g of soil samples was diluted with 2 mL of PBS solution in a 20 mL headspace glass vials (Agilent Technologies). The headspace vials were tightly capped with Teflon faced rubber liner cap and subjected for incubation for 72 h in dark at 25 °C. After incubation period the vials were equilibrated for 30 min at 60 °C using TriPlus RSH autosampler (Thermo Scientific, USA). The SPME fibre after activation in SSL injector was exposed and maintained in the vial headspace surface for 15 min. The volatile profile analysis was performed on GC-MS/MS (Trace 1310, TSQ 9000, Thermo Scientific, USA) with electron impact ionization (70 eV ionization energy). The separation was performed on Agilent HP-5MS capillary column (30 m x 0.25 mm, 0.25 µm) using helium as carrier gas with 1.2 mL $\cdot min^{-1}$ flow. SPME fibre was injected into the GC injection port and adsorbed volatiles in fibre were desorbed onto the column at 250 °C for 5 min in splitless mode. The volatile organic compounds were identified by comparison of their mass spectra with compounds corresponding mass spectra library (NIST/EPA/NIH, Chromeleon 7.2 CDS Software, Thermo Scientific, USA). Identified volatile organic compounds were expressed in percent as normalized amount of each volatile organic compound resulted after division of peak area of identified volatile organic compounds by total peak area of all identified volatile organic compounds.

# Statistical interpretation of data:

Differences in rhizosphere soil microbial community composition was investigated by principal component analysis (PCA) using Statistica 10 software (StatSoft, Germany). For the statistical analysis, the OD of each well after inoculation was subtracted from the OD after each measurement period during

incubation. Averages and standard deviations corresponding to each carbon sources were determined as samples were analysed in triplicate since Biolog EcoPlates contain three replicates of each carbon sources. Average well colour development (AWCD), Richness (S), Shannon's diversity index (H') and Shannon's evenness index (E) were determined according with formulas presented by Sofo and Ricciuti (2019). All these parameters were calculated separately for all incubation times. For Richness 0.25 value for optical density (OD) was set as threshold for positive response (Sofo and Ricciuti, 2019). ANOVA was conducted to assess the effect of studied pharmaceuticals on community level utilization of carbon sources. Assumption of homogeneity of variance and test for normality of distributions were verified applying Levene's test and Shapiro-Wilk's test using Statistica 10 software version (StatSoft, Germany). A level of $p = 0.05$ was considered to assume statistical significance. UpSetR diagram were performed according with Khan and Mathelier (2017).

# Results

## Rhizosphere microbial community abundance changes with contamination of NSAIDs

Microbiota as structure and abundance were monitored in *Lycopersicon esculentum* rhizosphere soils with and without artificially contamination with commonly consumed NSAIDs for 60 days assay period. The total microbial biomass was expressed as the sum of PLFAs which concentrations in control samples (without contamination) and those contaminated ranged between 165.6 and 240 $nmol \cdot g^{-1}$ dry weight soil during the assay (Fig. 2). The control rhizosphere soil recorded higher values of PLFA ($p \leq 0.05$) with concentration ranges of 184.8–240 $nmol \cdot g^{-1}$ dry weight soil. In soils contaminated with NSAIDs the microbial biomass in *Lycopersicon esculentum* rhizosphere soils has the following pattern: 187.9–215.4 (ketoprofen contamination) > 186.7–201.2 (diclofenac contamination) > 165.6–182.7 (ibuprofen contamination) $nmol \cdot g^{-1}$ dry weight soil, respectively (Fig. 2).

The PCA of the 48 PLFAs data (Fig. 3) indicated that rhizosphere soil microbial community abundance was markedly affected by soil contamination with NSAIDs but poor differentiation between control and contamination with ibuprofen was observed, indicated by their closest scores along the first principal component (PC1) and second principal component (PC2). The first two components, PC1 and PC2 explained 44.92 % and 27.29 % of the total variance in PLFAs abundance. PC1 axis differentiated contamination with diclofenac and ketoprofen but not differentiate controls by contamination with ibuprofen, whereas PC2 axis did not differentiated well control samples by contamination with specific NSAIDs.

## Rhizosphere microbial community structure changes in time with contamination of NSAIDs

Rhizosphere microbiota structure abundance differed through assays sampling period in all studied cases. Starting from day one until day thirty of the assay an increasing tendency was observed, followed by a stabilization until day sixty of the assay (Fig. 2).

*Lycopersicon esculentum* rhizosphere soils presented a bacterial dominance (Table 3) in all studied assay. The ratios of fungi to bacteria (F/B), gram negative bacteria to gram positive bacteria (G-/G+), aerobes bacteria to anaerobes bacteria (AerB/AnB), and ectomycorrhizal fungi/saprotrophic fungi (Ecto/Sapro) are presented in Table 3. According with that it was observed that among bacterial community higher dominance was observed in case of gram negative and aerobic bacteria ones. The average amount of fungal/bacterial ratio among contaminated soils with specific NSAIDs and period of exposure revealed the following pattern: control > contamination with ibuprofen > contamination with ketoprofen > contamination with diclofenac. Compared with control the average amount of ratio of gram negative/gram positive bacteria was higher in rhizosphere soil contaminated with ibuprofen and lower in case of diclofenac contamination (Table 3). Similarly, the ratio of aerobic/anaerobic bacteria presented an increasing tendency compared with that of control, with the following pattern: control < contamination with ibuprofen < contamination with ketoprofen < contamination with diclofenac (Table 3).

Table 3
Microbiota phenotypic structure components ratio variation among contamination

| NSAIDs | Day | Fungi/ Bacteria | Gram (-)/ Gram (+) | Aerobes/ Anaerobes | Ectomycorrhizal /Saprotrophic |
|---|---|---|---|---|---|
| Control | 1 | 0.131 | 2.585 | 2.157 | 0.695 |
| | 7 | 0.128 | 2.422 | 3.286 | 0.496 |
| | 30 | 0.122 | 2.151 | 3.090 | 0.669 |
| | 60 | 0.121 | 2.518 | 3.274 | 0.614 |
| Ibuprofen | 1 | 0.135 | 4.926 | 2.111 | 0.702 |
| | 7 | 0.104 | 3.382 | 2.351 | 0.916 |
| | 30 | 0.080 | 3.165 | 3.211 | 0.849 |
| | 60 | 0.075 | 3.157 | 4.820 | 0.835 |
| Ketoprofen | 1 | 0.100 | 2.379 | 2.805 | 1.033 |
| | 7 | 0.090 | 1.887 | 3.747 | 1.441 |
| | 30 | 0.095 | 1.950 | 3.977 | 1.594 |
| | 60 | 0.099 | 1.946 | 4.386 | 2.360 |
| Diclofenac | 1 | 0.094 | 2.764 | 4.063 | 0.722 |
| | 7 | 0.079 | 2.113 | 5.825 | 0.564 |
| | 30 | 0.085 | 1.604 | 6.740 | 0.555 |
| | 60 | 0.087 | 1.359 | 9.143 | 0.499 |

Principal component analysis was used as a data reduction strategy to infer correlations between rhizosphere microbiota community structure abundance evolution in time for each contamination experiment (Fig. 4a-d). The first and second components of the principal coordinate accounted for 56.86 % and 19.91 % of the total variance of the microbiota structure abundance in time for no contamination (control, Fig. 4a), 63.34 % and 11.8 % for diclofenac contamination (Fig. 4b), 62.36 % and 21.61 % for ketoprofen contamination (Fig. 4c), and 61.77 % and 17.44 % for ibuprofen contamination (Fig. 4d).

In control assay, aerobes and sulphur reducing bacteria PLFAs abundance was differentiated by first axes with 56.86 % by the rest of rhizosphere microbiota community PLFAs in day 1. The second axes differentiated gram negative, nitrogen reducing bacteria and actinomycetes by the rest of the rhizosphere community with 16.91 % (Fig. 4a). For diclofenac assay, fungi, anaerobes bacteria, gram negative bacteria and ectomycorrhizal fungi clearly differentiated by the rest of community through first axes

(63.34 %) in day 1 and 7 of the assay (Fig. 4b). Through contamination with ketoprofen differentiation within sulphur reducing bacteria, aerobes bacteria, gram negative bacteria, actinomycetes, methanotrophs bacteria, anaerobes bacteria, saprotrophic fungi and fungi was observed through first axes in day 1 and 7 of the assay with 63.36 % (Fig. 4c). In assay of ibuprofen impact on rhizosphere microbiota community structure abundance the first axes of PCA differentiated with 61.77 % nitrogen and sulphur reducing bacteria, actinomycetes, anaerobes bacteria, fungi, arbuscular mycorrhizal fungi, saprotrophic fungi and methanotrophs bacteria in day 1 and 7 of assay by the rest of the community (Fig. 4d).

# Rhizosphere microbial community level physiological profile changes in time with contamination of NSAIDs

Rhizosphere microbiota potential metabolic activity can be inferred by the AWCD where highest value of AWCD indicate a higher metabolic activity of microorganisms (Table 4). We performed analysis after very-short term exposure (day 1), short term exposure (day 7), midterm exposure (day 30) and long-term exposure (day 60) at studied NSAIDs. Based on the indices of the carbon sources consumption base metabolic diversity the highest score for functional richness (S) was determined control samples (ranged between 13 to 20) followed by samples contaminated with diclofenac (Table 4) although minor differentiation was obtained for Shannon’s diversity index (H’) and Shannon’s evenness index (E) among control and contaminated samples.

Table 4
Average well colour development (AWCD), Richness (S), Shannon's diversity index (H') and Shannon's evenness index (E) variation among contamination

| NSAIDs | Day | AWCD | S | H | E |
|---|---|---|---|---|---|
| Control | 1 | 0.256 ± 0.011 | 16.667 ± 0.577 | 3.199 ± 0.027 | 1.138 ± 0.006 |
| | 7 | 0.242 ± 0.007 | 13.333 ± 1.155 | 3.041 ± 0.016 | 1.176 ± 0.035 |
| | 30 | 0.296 ± 0.002 | 17 ± 0.00 | 3.212 ± 0.006 | 1.134 ± 0.002 |
| | 60 | 0.317 ± 0.003 | 20.333 ± 1.155 | 3.213 ± 0.004 | 1.067 ± 0.022 |
| Ibuprofen | 1 | 0.192 ± 0.003 | 9 ± 0.00 | 3.025 ± 0.02 | 1.377 ± 0.009 |
| | 7 | 0.245 ± 0.006 | 11.333 ± 0.577 | 3.162 ± 0.061 | 1.303 ± 0.011 |
| | 30 | 0.265 ± 0.005 | 13.00 ± 1.732 | 3.224 ± 0.012 | 1.262 ± 0.057 |
| | 60 | 0.258 ± 0.002 | 12.00 ± 0.00 | 3.203 ± 0.002 | 1.289 ± 0.001 |
| Ketoprofen | 1 | 0.237 ± 0.003 | 14.67 ± 0.577 | 3.15 ± 0.008 | 1.173 ± 0.019 |
| | 7 | 0.246 ± 0.004 | 15.0 ± 0.00 | 3.126 ± 0.026 | 1.154 ±0.010 |
| | 30 | 0.237 ± 0.002 | 13.0 ± 0.00 | 3.161 ± 0.003 | 1.232 ± 0.001 |
| | 60 | 0.234 ± 0.001 | 11.33 ± 0.577 | 3.121 ± 0.003 | 1.286 ± 0.025 |
| Diclofenac | 1 | 0.251 ± 0.009 | 13.0 ± 0.00 | 3.141 ± 0.020 | 1.225 ± 0.072 |
| | 7 | 0.271 ± 0.006 | 13.667 ± 0.577 | 3.041 ± 0.108 | 1.163 ± 0.037 |
| | 30 | 0.275 ± 0.003 | 15.333 ± 1.155 | 3.134 ± 0.010 | 1.149 ± 0.029 |
| | 60 | 0.273 ± 0.006 | 15.667 ± 1.155 | 3.102 ± 0.020 | 1.129 ± 0.025 |

Carbon source consumption increased with time of assay in case of control samples and those contaminated with diclofenac. This suggests that rhizosphere microbiota under contamination with diclofenac consume higher amount of carbon although do not consume a larger variety of its sources. In case of contamination with ibuprofen and ketoprofen the consumption of carbon source presents a decreasing tendency after day 30 of the assay (Table 4). Compared with PLFA data we supposed that rhizosphere communities are resilient at broad but not fine phenotypic levels: abundance of gram-negative bacteria, anaerobes bacteria, actinomycetes and arbuscular mycorrhizal fungi abundance under contamination with diclofenac decrease with approximately 35.5, 54.7, 53 and 49.4 %, respectively, compared with control.

Our heat map analysis (Fig. 5.) showed that in control samples poor consumption was determined after very-short term period of assay for glycyl-glutamic acid, d-glucosaminic acid, glucose-1-phosphate, d-cellobiose, i-erythritol, α-cyclodextrin and l-phenylalanine while after long-term assay period increase on tetrazolium reduction was detected for n-acetyl-d-glucosamine, d-glucosaminic acid, d-cellobiose, i-

erythritol, 2-hydroxy benzoic acid, α-cyclodextrin, and d-mannitol. Relative constant consumption during entire assay period was registered in control samples for carbon sources as carboxylic acids (γ-hydroxybutyric acid, d-malic acid, α-ketobutyric acid), carbohydrates (β-methyl-d-glucoside and d-l-lactose) and others as 4-hydroxy benzoic acid, phenylethylamine and l-arginine.

Towards to control samples, under exposure with diclofenac a decreased tendency in time was observed for phenolic compounds ($F = 4.924$, $p = 0.004$) and amines ($F = 18.5$, $p < 0.0001$) while carbohydrates ($F = 4.568$, $p = 0.043$) and carboxylic acids ($F = 33.888$, $p < 0.0001$) increased until end of the assay period. In rhizosphere soil contaminated with ibuprofen a decrease of consumption for d-galacturonic acid and n-acetyl d-glucosamine was recorded. Compared with control, increase in consumption of carboxylic acids ($F = 10.04$, $p < 0.001$) and amino acids ($F = 23.504$, $p < 0.0001$) was observed at the end of the assay. No influence on tetrazolium reduction rate was determined for carbon substrates as pyruvic acid methyl ester, 4-hydroxybenzoic acid, phenylethylamine, putrescine, α-keto butyric acid, α-d-lactose, and d-mannitol. In assays of exposure with ketoprofen poor consumption was recorded in case of pyruvic acid methyl ester and d-mannitol substrates. Towards to control samples assay, increase of amino acids (glycyl-l-glutamic acid, l-threonine, l-arginine) consumption was observed until the end of the assay ($F = 13.009$, $p < 0.0001$). Polymer substrates consumption decreased in time compared with control samples ($F = 20.547$, $p = 0.012$).

In overall, the rhizosphere communities in the presence of different NSAIDs changed the carbon consumption for each substrate classes. In soils exposed to ketoprofen and diclofenac a significant decrease in utilization of carbon substrates was observed compared with soil exposed to ibuprofen or control sample (Table 4, Fig. 5).

# Rhizosphere microbial community emitted volatile organic compounds changes with contamination of NSAIDs

We performed a VOCs analysis by SPME-GC-MS in all control and contaminated rhizosphere soils through exposure assay. Volatile compounds belonging to alcohol, aromatic compounds, ketone, terpene, organic acids, aldehyde, sulphur compounds, esters, alkane, nitrogen compounds, alkene and furans were detected in rhizosphere soil samples. Terpene, ketone, alcohol, aromatic compounds, organic acids, and alkane were the most abundant compound classes (Table 5). In control samples the highest alcohol compound was hexan-1-ol followed by 1-octen-3-ol with the average amount through assays period of 2.87 and 2.64 %, respectively. Benzaldehyde was the highest aromatic compounds identified in control samples and those contaminated with diclofenac (3.38 %) and ibuprofen (6.61 %). In case of ketoprofen contamination phenol was the highest aromatic compound released by rhizosphere microbiota (3.25 %). Among ketones the prevalent compounds were decane-2-one (3.41 %, diclofenac contamination), octan-3-one (control and ketoprofen contamination, 2.84 %) and decan-2-one (ibuprofen contamination, 3.34 %). Terpineol was determined in similar amount in case of control sample as well in case of contamination with ketoprofen (5.55 %). Compounds as geranyl acetone (5.84 %) and germacradien-1l-ol (4.89 %) were the most abundant released terpenes by rhizosphere microbiota under contamination with ibuprofen and

diclofenac, respectively. Butanoic acid was the most prevalent organic acid in all studied rhizosphere soils, with highest amount identified in rhizosphere soils under contamination with diclofenac (4.32 %), and followed by control and ketoprofen contaminated soil samples (3.44 %) and that with contamination with ibuprofen (2.84 %).

Table 5
Distribution of volatile organic compounds (%) produced in rhizosphere soil samples (control and NSAIDs contaminated)

| Volatile organic compounds | Control | Ketoprofen | Ibuprofen | Diclofenac |
|---|---|---|---|---|
| Alcohol | 12 | 13 | 18 | 12 |
| Aromatic compounds | 11 | 11 | 15 | 11 |
| Ketone | 13 | 14 | 11 | 12 |
| Terpene | 18 | 18 | 14 | 12 |
| Organic acids | 12 | 12 | 9 | 15 |
| Aldehyde | 6 | 8 | 6 | 8 |
| Sulphur compounds | 2 | 1 | 6 | 5 |
| Ester | 4 | 4 | 5 | 5 |
| Alkane | 12 | 11 | 10 | 11 |
| Nitrogen compounds | 5 | 5 | 4 | 3 |
| Alkene | 4 | 3 | 2 | 6 |
| Furans | 1 | 0 | 0 | 0 |

To identify volatile organic compounds specific or shared regulations, we compared the sets of up-regulated volatile organic compounds obtained for each exposure case and time of assays (Fig. 6).

Between 16 to 11 up-regulated volatile organic compounds responded specifically to the presence of NSAIDs. Among the remaining volatile organic compounds that were up regulated by at least two different pharmaceutical compounds, the highest intersection size was found to increase once with duration of assay (19 volatile organic compounds representing 37 % of the total number of up regulated volatile organic compounds in rhizosphere soils after 60 days of assay, **Fig. 6.d**). Interactions varied in time with assay duration, volatile organic compounds that were upregulated by control and at least two different NSAIDs the highest size was found after midterm exposure (day 30, **Fig. 6.c**) and short-term exposure (day 7, **Fig. 6.b**) of the assay. Similarities of volatile organic compounds in assays at very-short term exposure (day 1) were found for 2 up-regulated volatile organic compounds when rhizosphere microbiota was exposed to ibuprofen and ketoprofen and other 2 up-regulated volatile organic compounds were found when exposure was for ibuprofen and diclofenac (Fig. 6.**a**). In case of short-term exposure assay (day 7) similarities of volatile organic compounds were found for 6 up-regulated volatile

organic compounds when rhizosphere microbiota was exposed to ibuprofen and diclofenac (Fig. 6.**b**) while at mid-term exposure assay 5 up-regulated volatile organic compounds when rhizosphere microbiota was exposed to ibuprofen and diclofenac and 6 up-regulated volatile organic compounds when rhizosphere microbiota was exposed to ibuprofen and ketoprofen (Fig. 6.**a**).

# Discussions

In this study we investigated the impact of commonly reported NSAIDs in environment on *Lycopersicon esculentum* rhizosphere microbiota. Investigation targeted the effect of diclofenac, ketoprofen and ibuprofen on *Lycopersicon esculentum* rhizosphere microbiome abundance, phenotypic structure and metabolic activity as carbon source consumption and emitted volatile organic compounds. Currently there are poor information related to the effects of commonly reported NSAIDs on the rhizosphere microbiome abundance and metabolic activity, most the study referring to antibiotics impact on bulk soil microbiome (Aleanizy et al., 2021; Wu et al., 2021; Aguilar-Romero et al., 2020; Shen et al., 2019;). Therefore, it was difficult to compare our results with those obtained by other authors.

In general, the reported data showed differentiated effects of pharmaceuticals on bulk soil microorganisms, data differing for each pharmaceutical in part, concentration of pharmaceuticals and time of exposure (Wu et al., 2021; Frkova et al., 2020; Shen et al., 2019). Also, most studies involved assays on isolated microorganisms (Aleanizy et al., 2021; Dai et al., 2019; Oliviera et al., 2019) and not on rhizosphere microbiome. However, these data often showed changes in strain vitality or their enzymatic activity. Obtained data in study performed on *Pseudomonas sp.*, by Aleanizy et al. (2021) showed a reduction in abundance, pyocyanin production, and specific enzymatic activity (protease, DNase) after exposure to antibiotics as azithromycin, piperacillin/tazobactam, and cefepime. Dai et al. (2019) has shown in their study that *Pseudomonas aeruginosa* biofilm formation and adherence acidity significantly reduced after exposure at ibuprofen. Similarly, Oliviera et al. (2019) reposted that *Staphylococcus aureus* growth in planktonic and biofilm states, was controlled by ibuprofen exposure at different concentration. In our study we observed that *Lycopersicon esculentum* rhizosphere microbiome abundance decreased especially under exposure to ibuprofen and diclofenac. Changes in phenotypic structure was also observed in our experimental data, especially in case of fungal/bacteria, gram positive/gram negative bacteria and aerobic/anaerobic bacteria ratios. Our data were following those reported by Cycon et al., (2016), Paje et al., (2002), and Dastidar et al., (2000).

Considering the rhizosphere microbiome emitted volatile organic compounds, differentiated pattern was observed in time, depending also by the NSAIDs used in exposure experiment. In case of diclofenac exposure assessment, our data reveal increasing tendency with time in case of specific alcohol compounds (2-methyl-butan-1-ol, hexan-1-ol, and 1-octen-3-ol), aromatic compounds (benzaldehyde, 1-methoxy-4-methylbenzene, and phenol) and germacradien-1l-ol. In cased of alcohol compounds similar pattern was observed even when contamination was performed with ketoprofen. The remaining volatile organic compounds, either decreased during the assay time, either presented an increasing tendency at the first half period of the assay after that decreased. Comparing these patterns with those reported in

literature, we found that Zhou et al., (2019) in their study on extracellular polymeric substances observed and increase once with exposure dose for ibuprofen. However, in all contamination assessment different pattern of volatile organic compounds emission were founded. Because volatile organic compounds are essential for above and belowground diversity; it becomes important to understand how these are affected by the presence of certain pharmaceuticals. Similarly, rhizosphere microbiome carbon consumption pattern differed also once with contamination with studied NSAIDs. Towards to control samples, in soils exposed to ketoprofen and diclofenac carbon substrates consumption was lower. Minor changes were observed in case of exposure to ibuprofen.

# Conclusions

Investigation of the impact of non-steroidal anti-inflammatory drugs on rhizosphere microbiota is essential to assessing their ecological risk. Results of artificial exposure experiments to diclofenac, ibuprofen and ketoprofen from this study clearly illustrate that presence of drugs could shape rhizosphere microbiota abundance and phenotypic structure. It demonstrates that also the metabolic activity of rhizosphere microbiome could be modified in time when they are in contact with pharmaceuticals. Therefore, to protect soil ecosystem services mediated by rhizosphere microbiome, presence of potential pharmaceuticals should be considered.

# Declarations

**Acknowledgements:** This work was funded by projects PN 19-18.01.01-18N/08.02.2019 and PREPARE SMIS 107874; 253/2.06.2020

**Funding**:

This work was funded by projects PN 19-18.01.01-18N/08.02.2019 and PREPARE SMIS 107874; 253/2.06.2020

**Conflicts of interest/Competing interests**:

Not the case

**Availability of data and material**:

Experimental raw data could be available under appropriate request of editors

**Code availability**:

Not the case

**Authors' contributions**:

All authors contributed equally at this manuscript realisation (experimental + writing)

**Ethics approval**:

Not the case

**Consent to participate**:

All authors give the consent to participate at this manuscript

**Consent for publication**

All authors give the consent for publication of this manuscript

# References


Aguilar-Romero, I., Romero, E., Wittich, R.M., Dillewijn, P. (2020) Bacterial ecotoxicity and shifts in bacterial communities associated with the removal of ibuprofen, diclofenac and triclosan in biopurification systems. Science of the Total Environment, 741:140461.

Ajilogba, C.F., Babalola, O.O. (2019) GC-MS analysis of volatile organic compounds from Bambara groundnut rhizobacteria and their antibacterial properties. World Journal of Microbiology and Biotechnology, 35:83.

Aleanizy, F.S., Alqahtani, F.Y., Eltayb, E.K., Alrumikan, N., Almebki, R., Alhossan, A., Almangour, T.A., AlQahtani, H. (2021) Evaluating the effects of antibiotics sub-inhibitory dose on Pseudomonas aeruginosa quorum sensing dependent virulence and its phenotypes. Saudi Journal of Biological Sciences, 28(1):550-559.

Alenzi, A., Hunter, C., Spencer, J., Roberts, J., Craft, J., Pahl, O., Escudero, A. (2021) Pharmaceuticals effect and removal, at environmentally relevant concentrations, from sewage sludge during anaerobic digestion. Bioresource Technology, 319:124102.

Ayukekbong, J.A., Ntemgwa, M., Atabe, A.N. (2017) The threat of antimicrobial resistance in developing countries causes and control strategies. Antimicrobial Resist Infect Control, 6:47.

Bhattacharyya, D., Lee, Y.H. (2016) The bacterial community in the rhizosphere of Kimchi cabbage restructured by volatile compounds emitted from rhizobacterium Proteus vulgaris JBLS202. Applied Soil Ecology, 105:48-56.

Blight, E.G., Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37:911-917.

Brown, R.W., Bull, I.D., Journeaux, T., Chadwick, D.R., Jones, D.L. (2021) Volatile organic compounds (VOCs) allow sensitive differentiation of biological soil quality. Soil Biology and Biochemistry, 156:108187.

Chaturvedi, P., Shukla, P., Giri, B.S., Chowdhary, P., Chandra, R., Gupta, P., Pandey, A. (2021) Prevalence and hazardous impact of pharmaceutical and personal care products and antibiotics in environment: a review on emerging contaminants. Environmental Research, 194:110664.

Chessa, L., Pusino, A., Garau, G., Mangia, N.P., Pinna, M.V. (2016) Soil microbial response to tetracycline in two different soils amended with cow manure. Environmental Science and Pollution Research, 23:5807-5817.

Courtier, A., Cadiere, A., Roig, B. (2019) Human pharmaceuticals: why and how to reduce their presence in the environment. Current Opinion in Green and Sustainable Chemistry, 15:77-82.

Cycon, M., Borymski, S., Zolnierczyk, B., Piotrowska-Seget, Z. (2016) Variable effects of Non-steroidal Anti-inflammatory Drugs (NSAIDs) on selected biochemical processes mediated by soil microorganisms. Frontiers in Microbiology, 7:1969.

Dai, L., Wu, T., Xiong, Y., Ni, H., Ding, Y., Zhang, W., Chu, S., Ju, S., Yu, J. (2019) Ibuprofen-mediated potential inhibition of biofilm development and quorum sensing in Pseudomonas aeruginosa. Life Sciences, 237:116947.

Dastidar, S. G., Ganguly, K., Chaudhuri, K., Chakrabarty, A. N. (2000). The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. *Int. J. Antimicrob. Agents* 14 249–251.

Duan, Y.P., Meng, X.Z., Wen, Z.H., Chen, L. (2013) Acidic pharmaceuticals in domestic wastewater and receiving water from hyper-urbanization city of China (Shanghai): environmental release and ecological risk. Environmental Science and Pollution Research, 20:108-116.

Fedaku, S., Alemayehy, E., Dewill, R., Bruggen, B. (2019) Pharmaceuticals in freshwater aquatic environments: a comparison of African and European challenge. Science of the Total Environment, 654:324-337.

Fincheira, P., Quiroz, A., Tortella, G., Diez, M.C., Rubilar, O. (2021) Current advances in plant-microbe communication via volatile organic compounds as an innovative strategy to improve plant growth. Microbiological Research, 247:126726.

Frostegard, A., Baath, E. (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils, 22:59-65.

Frostegard, A., Tunlid, A., Baath, E. (2011) Use and misuse of PLFA measurements in soils. Soil Biology and Biochemistry, 43:1621-1625.

Frkova, Z., Vystavna, Y., Koubova, A., Grabicova, K., Grabic, R., Kodesova, R., Chronakova, A. (2020) Microbial response to selected pharmaceuticals in agricultural soils: microcosm study on the roles of soil, treatment, and time. Soil Biology and Biochemistry, 149:107924.

Gama-Rodrigues, A.C. (2011) Soil organic matter, nutrient cycling, and biological dinitrogen-fixation in agroforestry systems. Agroforestry Systems, 81:191-193.

Garcia-Rodriquez, A., Matamores, V., Fontas, C., Salvado, V. (2014) The ability of biologically based wastewater treatment systems to remove emerging organic contaminants – a review. Environmental Science and Pollution Research, 21:11708-11728.

Gianinazzi, S., Gollotte, A., Binet, M.N., Tuinen, D., Redecker, D., Wipf, D. (2010) Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza, 20:519-530.

Grabicova, K., Grabic, R., Fedorova, G., Stanova, A.V., Blaha, M., Randak, T., Brooks, B.W., Zlabek, V. (2020) Water reuse and aquaculture: pharmaceutical bioaccumulation by fish during tertiary treatment in wastewater stabilization pond. Environmental Pollution, 267:115593.

Hofmann, K., Lamprecht, A., Pauli, H., Illmer, P. (2016) Distribution of prokaryotic abundance and microbial nutrient cycling across a high alpine altitudinal gradient in the Austrian Central Alps is affected by vegetation, temperature, and soil nutrients. Microbial Ecology, 72:704-716.

Imade, E.E., Babalola, O.O. (2021) Biotechnological utilization: the role of Zea mays rhizospheric bacteria in ecosystem sustainability. Applied Microbiology and Biotechnology, 105(3):1-14.

Joergensen, R.G., Wichern, F. (2008) Quantitative assessment of the fungal contribution to microbial tissue in soil. Soil Biology and Biochemistry, 40:2977-2991.

Khan, A., Mathelier, A. (2017) Intervene: a tool for intersection and visualization of multiple gene genomic region sets. BMC Bioinformatics, 18:287.

Kamat, S., Marathe, P., Tripathi, R., Raut, S., Khatri, N. (2020) Over-the-counter medicines: global perspective and Indian scenario, J. Postgrad. Med. 66:1-28.

Kang, W., Deng, X., Zhao, Z. (2007) Role of canopy interception on water and nutrient cycling in Chinese fir plantation ecosystem. Frontiers of Forestry in China, 2:448-452.

Kovacs, E.D., Silaghi-Dumitrescu, L., Kovacs, M.H., Roman, C. (2021) Determination of the uptake of ibuprofen, ketoprofen, and diclofenac by tomatoes, radishes, and lettuce by gas chromatography-mass spectrometry (GC-MS). Analytical Lettters, 54(1-2):314-330.

Lindahl, V., Bakken, L.R. (1995) Evaluation of methods for extraction of bacteria from soil. FEMS Microbial Ecology, 16:135-142.

Liu, F., Wu, J., Ying, G.G., Luo, Z., Feng, H. (2012) Changes in functional diversity of soil microbial community with addition of antibiotics sulfamethoxazole and chlortetracycline. Applied Microbiology and Biotechnology, 95:1615-1623.

Lopez, S.M.Y., Pastorino, G.N., Balatti, P.A. (2021) Volatile organic compounds profile synthesized and released by endophytes of tomato (Solanum lycopersici L.) and their antagonistic role. Archieves of Microbiology, 203:1383-1397.

Madikizzela, M.L., Chimuka, L. (2017) Occurrence of naproxen, ibuprofen, and diclofenac residues in wastewater and river water of KwaZulu-Natal Province in South Africa. Environmental Monitoring and Assessment, 189:348.

Mejias, C., Santos, L.J., Aparicio, I., Alonso, E. (2021) Occurrence of pharmaceuticals and their metabolites in sewage sludge and soil: a review on their distribution and environmental risk assessment. Trends in Environmental Analytical Chemistry, 30:e00125.

Mordechaay, E.B., Mordehay, V., Tachitzky, J., Chefetz, B. (2021) Pharmaceuticals in edible crops irrigated with reclaimed wastewater: evidence from large survey in Israel. Journal of Hazardous Materials, 416:126184.

Moulineux, C.J., Gange, A.C., Connop, S.P., Newport, D.J. (2015) Are microbial communities in green roof substrates comparable to those in post-industrial sites? – a preliminary study. Urban Ecosystems, 18:1-19.

Oliviera, I.M., Borges, A., Borges, F., Simoes, M. (2019) Repurposing ibuprofen to control Staphylococcus aureus biofilm. European Journal of Medicinal Chemistry, 166:197-205.

Orlandini, V., Maida, I., Fondi, M., Perrin, E., Papaleo, M.C., Bosi, E., Pascale, D., Tutino, M.L., Michaud, L., Giudice, A.L., Fani, R. (2014) Genomic analysis of three sponge associated Arthrobacter Antactic strains, inhibiting the growth of Burkholderia cepacian complex bacteria by synthesizing volatile organic compounds. Microbial Research, 169(7-8):593-601.

Paje, M. L. F., Kuhlicke, U., Winkler, M., Neu, T. R. (2002). Inhibition of lotic biofilms by diclofenac.*Appl. Microbiol. Biotechnol.* 59 488–492.

Papaioannou, D., Koukoulakis, P.H., Papageorgiou, M., Lambropoulou, D.A., Kalavrouziotis, I.K. (2020) Investigation of pharmaceutical and personal care product interactions of soil and beets (Beta vulgaris L.) under the effect of wastewater reuse. Chemosphere, 236:124553.

Pino-Otin, R.M., Muniz, S., Val, J., (2017) Navarro E. Effects of 18 pharmaceuticals on the physiological diversity of edaphic microorganisms. Science of the Total Environment, 595:441-450.

Praveenkumarreddy, Y., Vimalkumar, K., Ramaswamy, B.R., Kumar, V., Singhai, R.K., Basu, H., Gopal, C.M., Vandana, K.E., Bhat, K., Udayashankar, H.K., Balakrishna, K. (2021) Assessment of non-steroidal anti-inflammatory drugs from selected wastewater treatment plants of Southwestern India. Emerging Contaminants, 7:43-51.

Quintelas, C., Mesquita, D.P., Torres, A.M., Costa, I., Ferreira, E.C. (2020) Degradation of widespread pharmaceuticals by activated sludge: kinetic study, toxicity assessment, and comparison with adsorption. Journal of Water Processes Engineering, 33:101061.

Ramirez-Guizar, S., Sykes, H., Perry, J.D., Schwalbe, E.C., Stanforth, S.P., Perez-Perez, C.I., Dean, J.R., (2017) A chromatographic approach to distinguish Gram-positive from Gram-negative bacteria using exogenous volatile organic compound metabolites. Journal of Chromatography A, 1501:79-88.

Ramires, P.F., Tavella, A.R., Escarrone, L.M., Volcao, L.M., Honscha, L.C., Brum, R.L., Silva, A.B., Silva Junior, F.M.R. (2021) Ecotoxicity of triclosan in soil: an approach using different species. Environmental Science and Pollution Research.

Rode, M.W. (1995) Aboveground nutrient cycling and forest development on poor sandy soil. Plant and Soil, 168:337-343.

Sanon, A., Andrianjaka, Z.N., Prin, Y., Thioulouse, B.J., Comte, G., Duponnois, R. (2009) Rhizosphere microbiota interferes with plant-plant interactions. Plant and Soil, 321:259-278.

Sebok, A., Vasanits-Zsigrai, A., Palko, Gy., Zaray, Gy., Molnar-Perl, I. (2008) Identification and quantification of ibuprofen, naproxen, ketoprofen and diclofenac present in wastewater, as their trimethylsilyl derivatives, by gas chromatography mass spectrometry. Talanta, 76(3):642-650.

Shen, Y., Stedtfeld, R.D., Guo, X., Bhalsod, G.D., Jeon, S., Tiedje, M.J., Li, H., Zhang, W. (2019) Pharmaceutical exposure changed antibiotic resistance genes and bacterial communities in soil-surface and overhead-irrigated greenhouse lettuce. Environmental International, 131:105031.

Sofo, A., Ricciuti, P. (2019) A standardized method for estimating the functional diversity of soil bacterial community by Biolog® EcoPlatesTM assay – The case study of a sustainable olive orchard. Applied Sciences, 9:4035.

Veuger, B., Pitcher, A., Schouten, S., Sinninghe Damste, J.S., Middelburg, J.J. (2013) Nitrification and growth of autotrophic nitrifying bacteria and Thaumarchaeota in the costal North Sea, Biogeoscience, 10:1775-1785.

Wang, L., Zhang, W., Wang, J., Zhu, L., Wang, J., Yan, S., Ahmad, Z. (2019) Toxicity of enrofloxacin and cadmium alone and in combination to enzymatic activities and microbial community structure in soil. Environmental Geochemistry and Health, 41:2593-2606.

Wu, W., Ma, M., Hu, Y., Yu, W., Liu, H., Bao, Z. (2021) The fate and impacts of pharmaceuticals and personal care products and microbes in agricultural soils with long term irrigation with reclaimed water. Agricultural Water Management, 251:106862.

Xu, J., Wu, L., Chen, W., Chang, C.A. (2010) Leaching potential of nonsteroidal anti-inflammatory drugs in soils. Environmental Toxicology and Chemistry 29(4):800-7.

Zhou, G., Li, N., Rene, R.E., Liu, Q., Dai, M., Kong, Q., (2019) Chemical composition of extracellular polymeric substances and evolution of microbial community in activated sludge exposed to ibuprofen. Journal of Environmental Management, 246:267-274.

# Figures

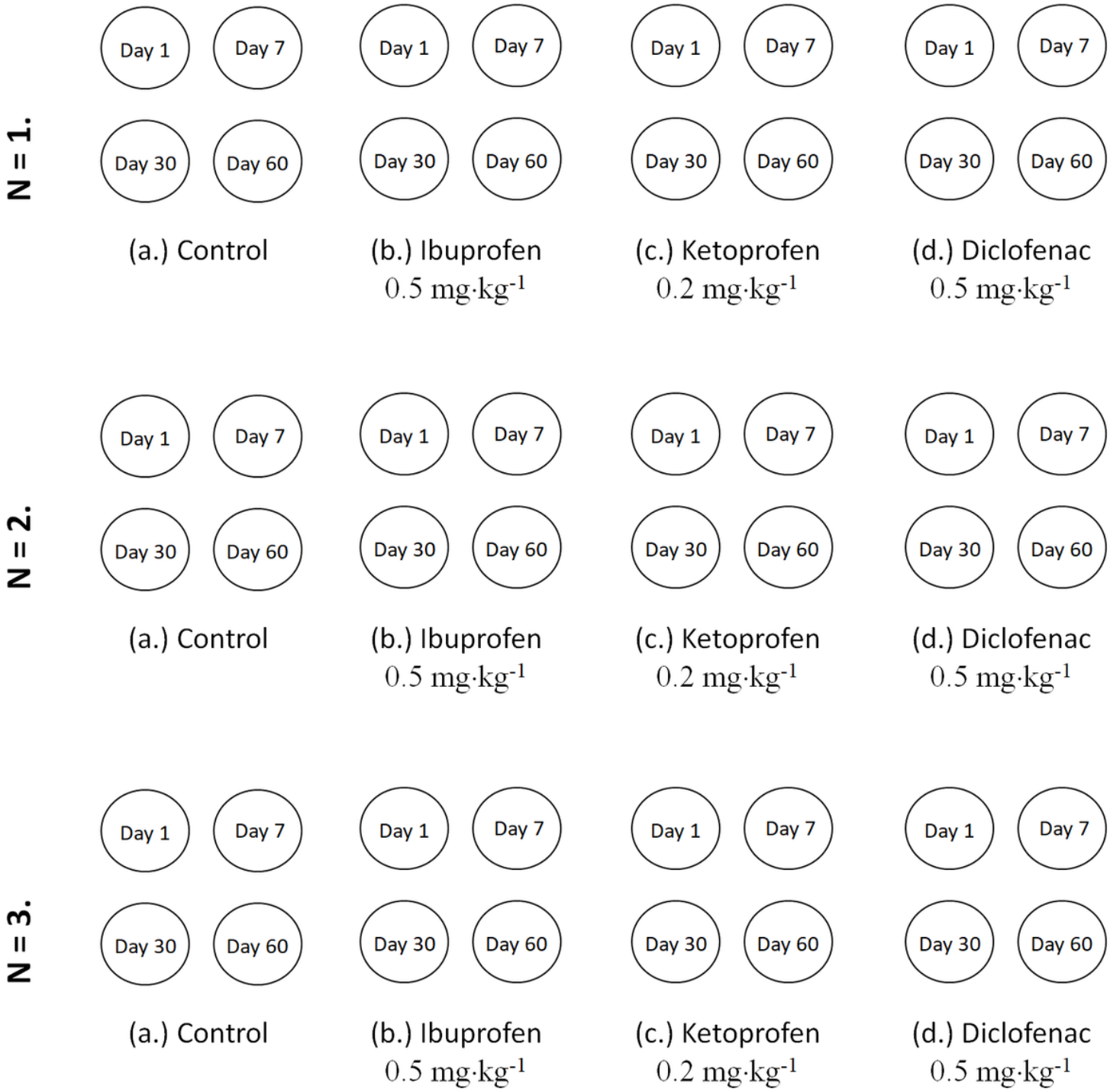
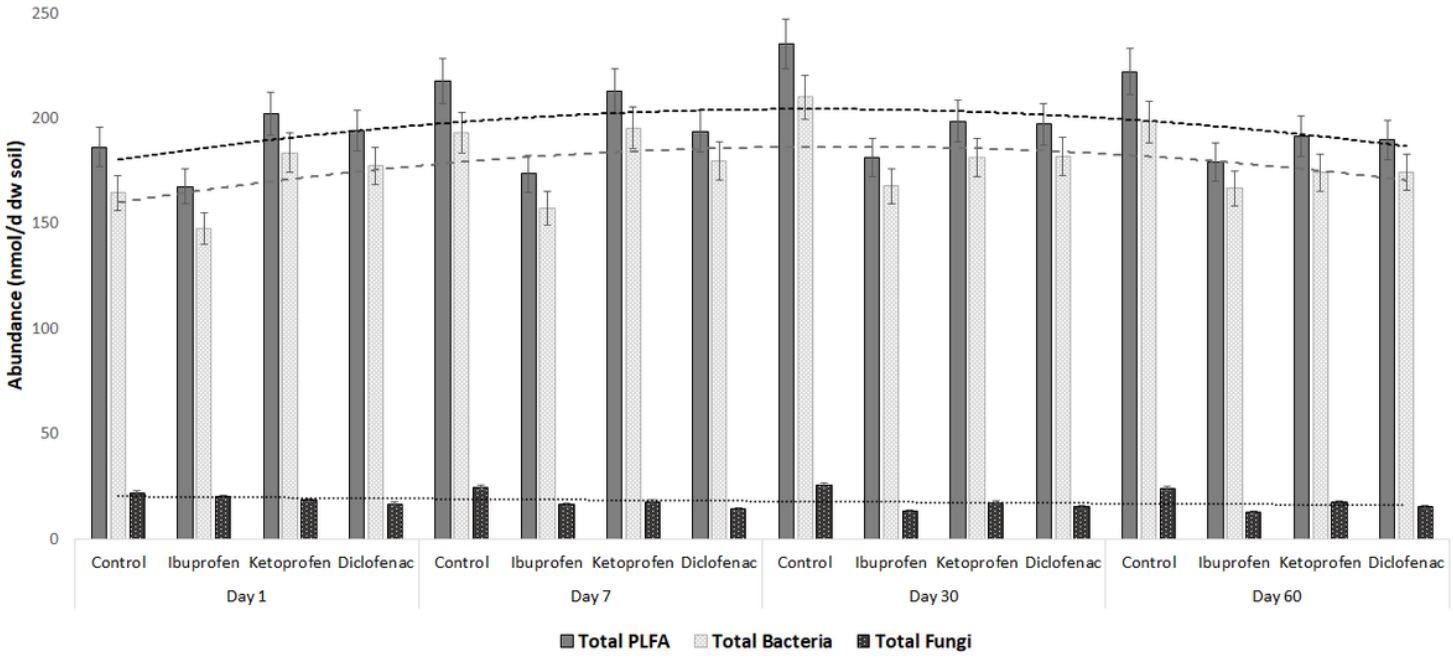



**Figure 1**

Schematic diagram of experiment set-up




## Figure 2

Soil microbiota abundance variation in rhizosphere soil during assay period

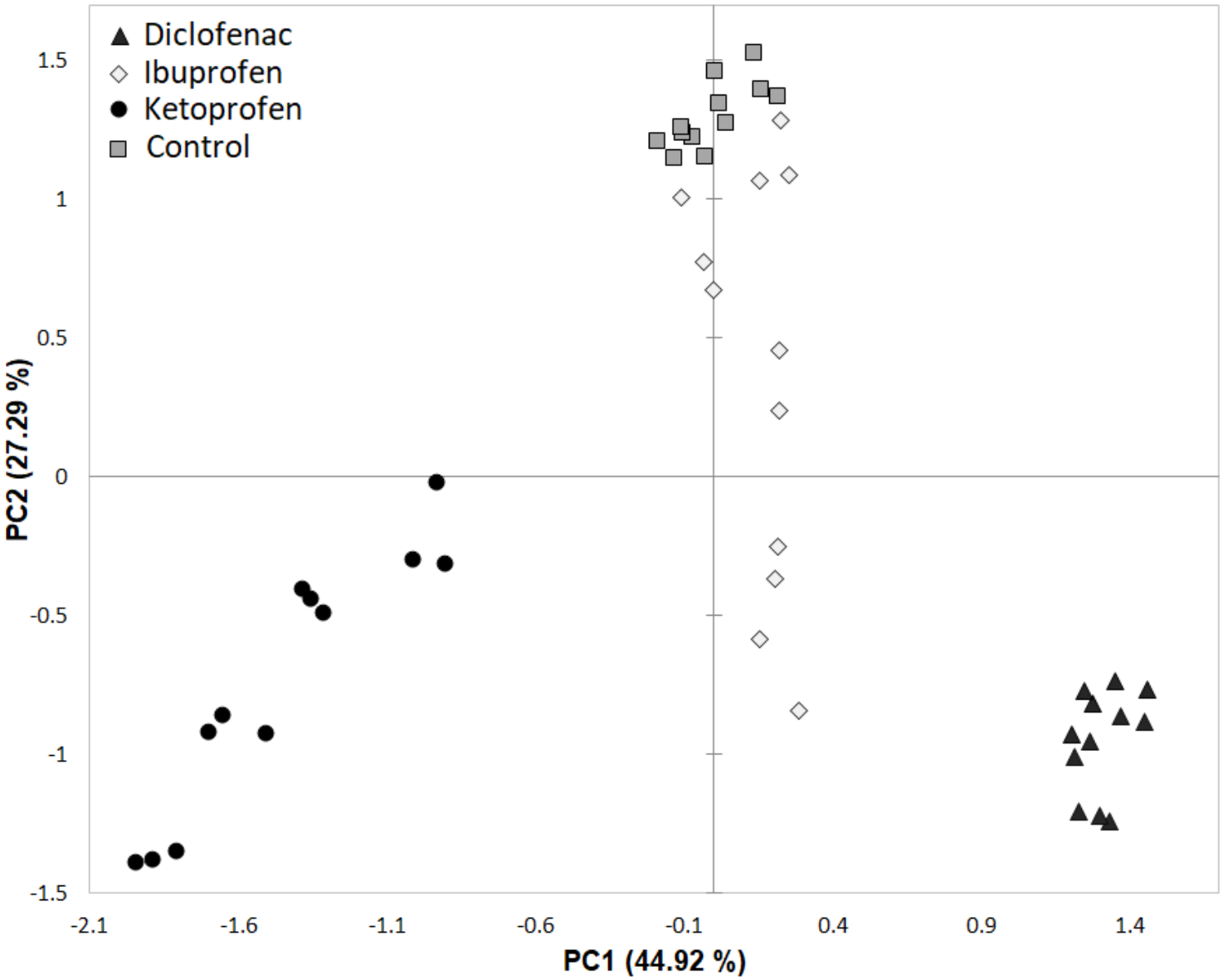
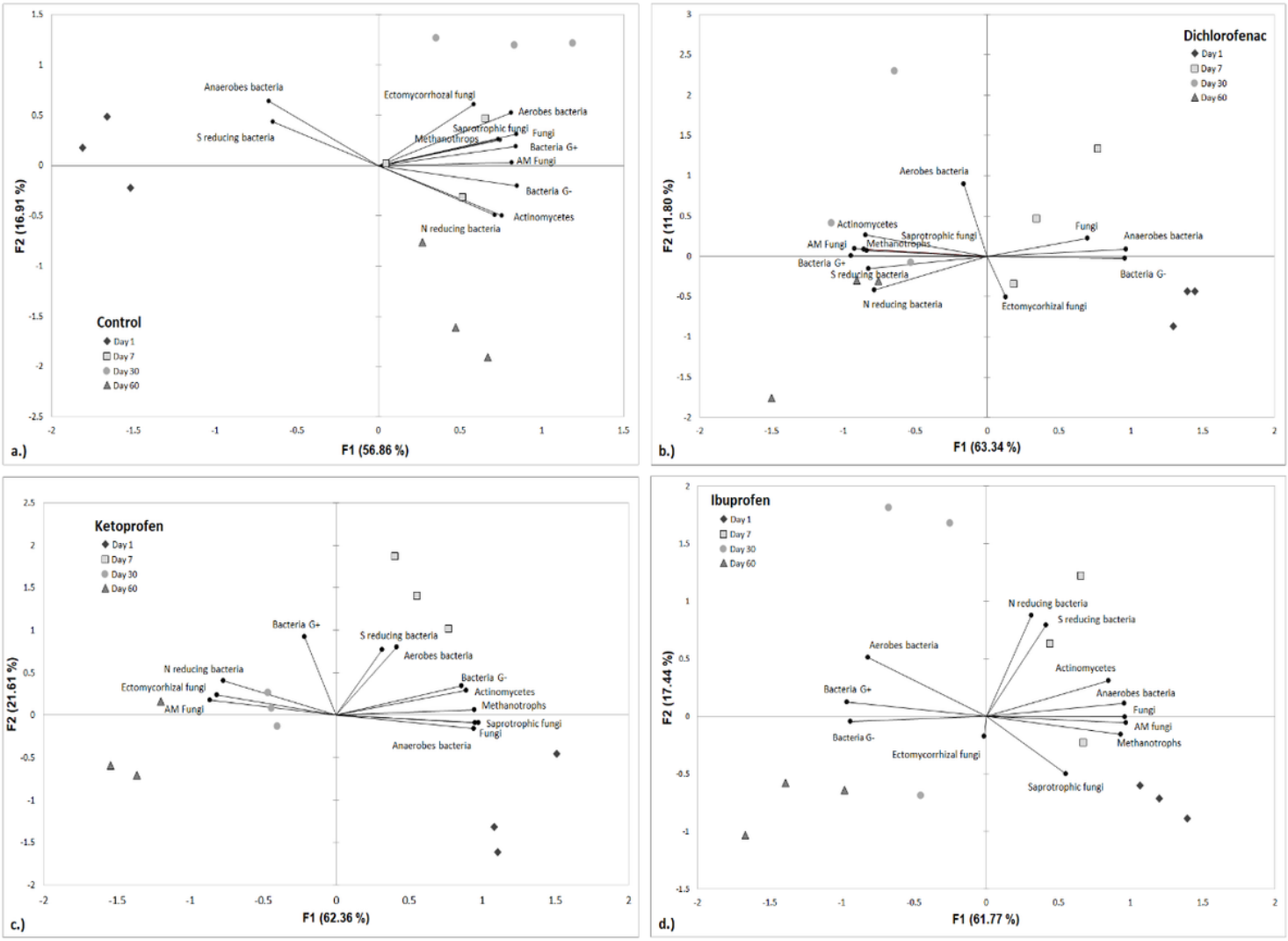


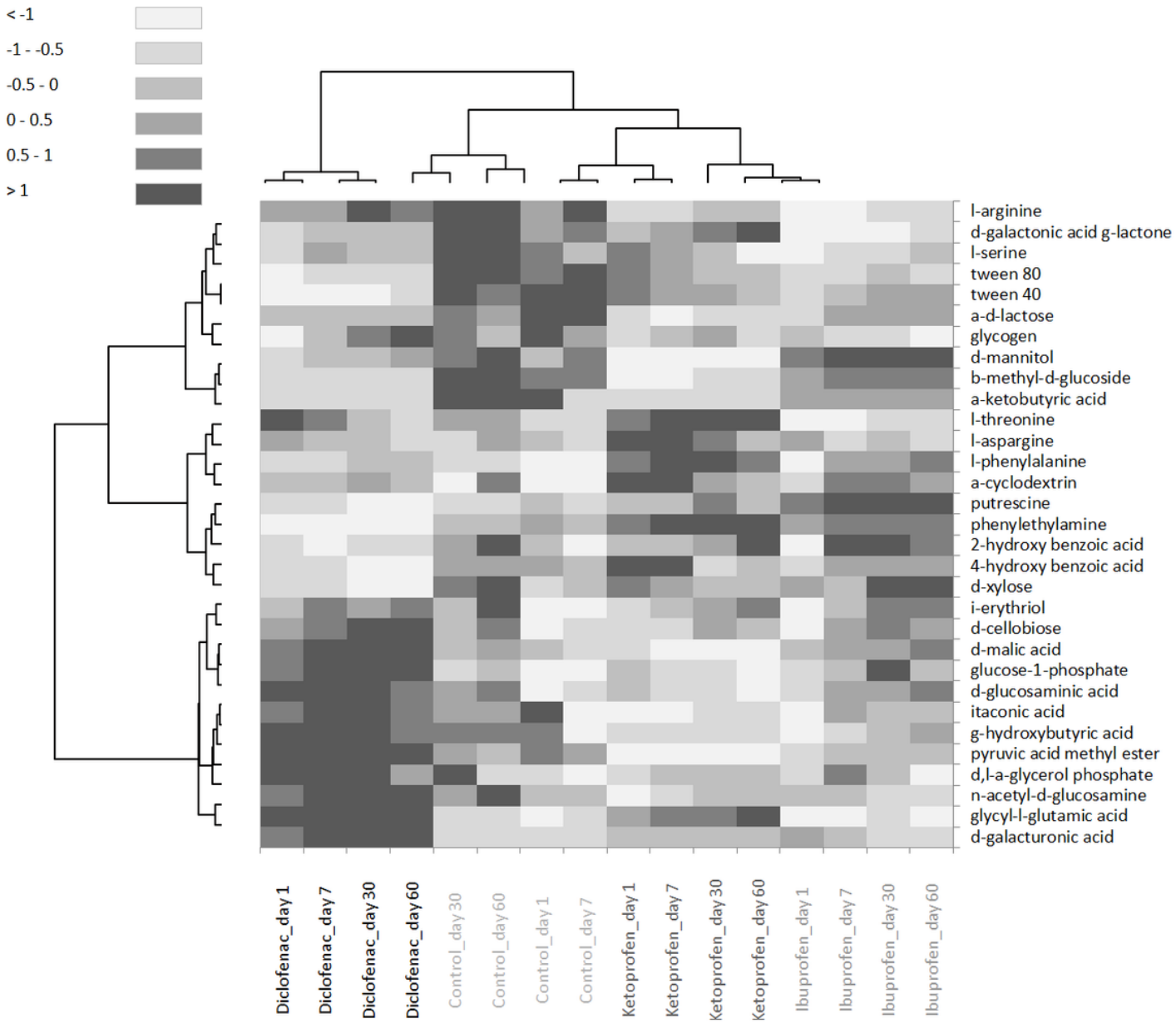

**Figure 3**

Principal component analysis (PCA) of rhizosphere PLFAs from control samples and those contaminated with different NSAIDs



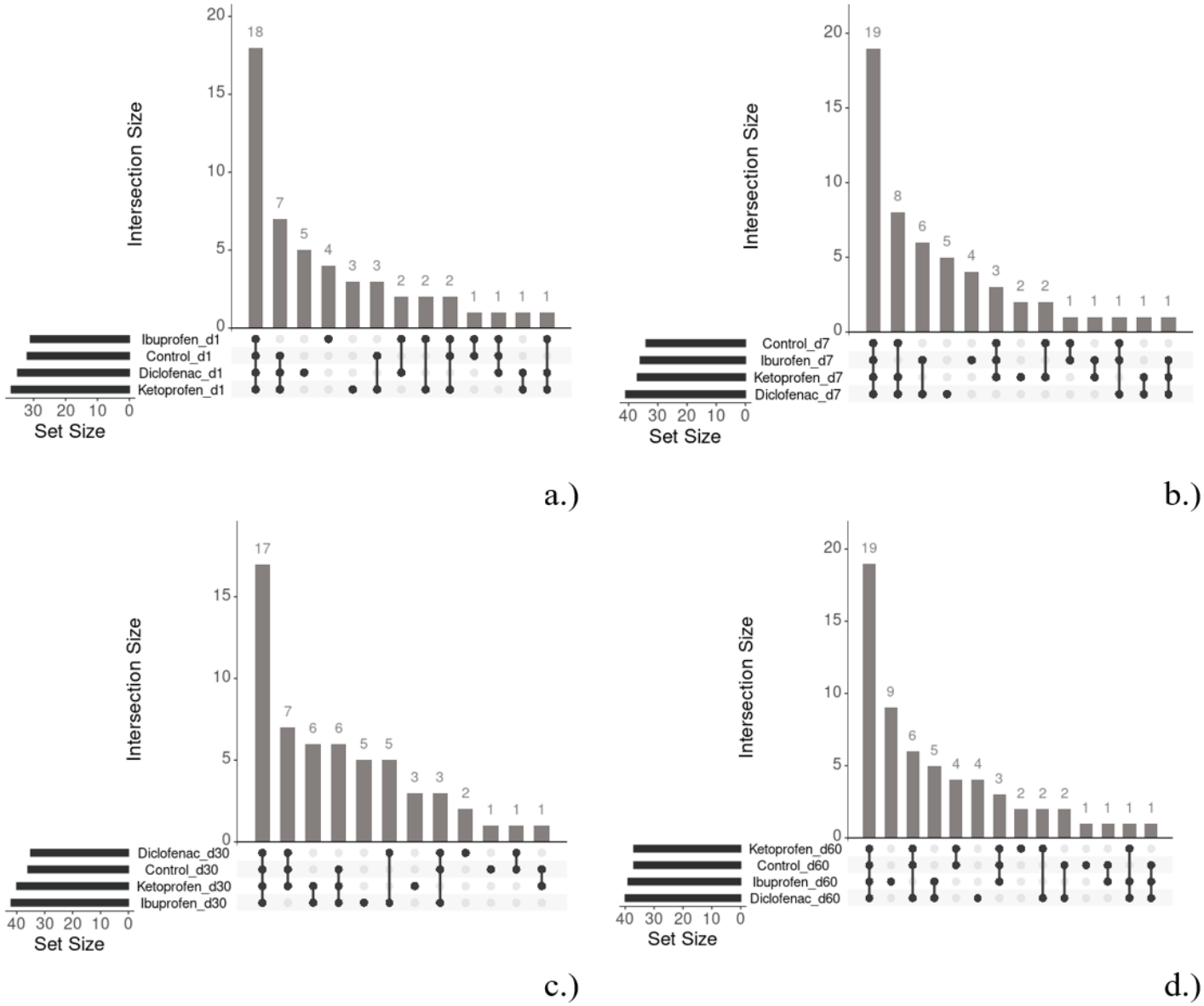

## Figure 4

Principal component analysis (PCA) of rhizosphere microbial community composition evolution in time for each contamination assay: a.) control, b.) diclofenac, c.) ketoprofen, d.) ibuprofen




## Figure 5

Heat map of rhizosphere microbial community level physiological profile changes in time for each contamination assay




## Figure 6

UpSet plot of interactions and the amounts of similar and different volatile organic compounds released by rhizosphere microbial community composition evolution in time for each contamination assay