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The fate of cigarette butts in different environments: Decay rate, chemical changes and ecotoxicity revealed by a 5-years decomposition experiment☆

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ABSTRACT

Cigarette butts (CBs) are the most common litter item on Earth but no long-term studies evaluate their fate and ecological effects. Here, the role of nitrogen (N) availability and microbiome composition on CBs decomposition were investigated by a 5-years experiment carried out without soil, in park grassland and sand dune. During decomposition, CBs chemical changes was assessed by both ^{13}C CPMAS NMR and LC-MS, physical structure by scanning electron microscope and ecotoxicity by *Aliivibrio fischeri* and *Raphidocelis subcapitata*. Microbiota was investigated by high-throughput sequencing of bacterial and eukaryotic rRNA gene markers. CBs followed a three-step decomposition process: at the early stage (~30 days) CBs lost ~15.2% of their mass. During the subsequent two years CBs decomposed very slowly, taking thereafter different trajectories depending on N availability and microbiome composition. Without soil CBs showed minor chemical and morphological changes. Over grassland soil a consistent N transfer occurs that, after de-acetylation, promote CBs transformation into an amorphous material rich in aliphatic compounds. In sand dune we found a rich fungal microbiota able to decompose CBs, even before the occurrence of de-acetylation. CBs ecotoxicity was highest immediately after smoking. However, for *R. subcapitata* toxicity remained high after two and five years of decomposition.

1. Introduction

Plastic pollution severely affects terrestrial and aquatic ecosystems, hence the current phase of human history has been referred as the "Plastic Age" (Thompson et al., 2009). Cigarette butts (CBs), by number, are the most common littered item on Earth, reaching an annual discard of several trillions (Novotny et al., 2009; Barnes, 2011). Accordingly, CBs are systematically recorded among the

Abbreviations: CB, cigarette butt; ^{13}C CPMAS NMR, Carbon-13 cross-polarization magic-angle-spinning nuclear magnetic resonance; LC-MS, Liquid chromatography-mass spectrometry; SEM, Scanning electron microscope; GLM, Generalized linear model; NMDS, Nonmetric multidimensional scaling.

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most common litter type in monitoring studies carried out in public areas and parks (Araújo and Costa, 2019). In urban areas, for instance, CBs are heterogeneously distributed reaching a density of 48 items m^{-2} (Green et al., 2014). CBs are very common also in coastal beaches, especially in urban sites and in the summer seasons (Ariza and Leatherman, 2012; Loizidou et al., 2018; Asensio-Montesinos et al., 2019).

Besides littering, the accumulation of CBs in different environments is mostly due to their slow decomposition rate. Most CBs are made of plasticized cellulose acetate, compressed and wrapped in an outer paper layer (Harris, 2011; Fig. S1). Cellulose acetate differs from simple cellulose for the large degree of acetate substitution (~2.45) that increases its filtering efficiency but makes it almost inaccessible to microbial decomposition (Rustemeyer, 2004). A substantial de-acetylation to a lower substitution degree (~1) by chemical hydrolysis is necessary to allow the microbial attack, but this process is slow under ambient conditions (Puls et al., 2011). The de-acetylation process can be promoted by UV radiation, as extensively shown in previous studies on pure cellulose acetate degradation. However, available evidence on the degradation of whole CBs in realistic ecological conditions is limited. According to a recent study (Bonanomi et al., 2015), CBs mass loss ranges between 20 and 30% after 2 years, but robust scientific data on long-term decomposition are lacking.

Microbial nitrogen (N) starvation is an additional factor that may explain the low CBs decomposition rates. Indeed, the low N content of CBs, corresponding to a C/N ratio of ~200, may limit microbial activity, hence inhibiting the decomposition process. The role of N availability in plant litter (i.e. leaf, root, and coarse wood debris) decomposition has been recognized as one of the most important together with temperature and soil moisture (Berg and McClaugherty, 2008). According to current knowledge, N becomes a limiting factor during the decay process if the organic substrate C/N ratio stays above the threshold of ~30–35, a condition where microbial activity is limited by N starvation if exogenous N inputs are lacking (Parton et al., 2007). Therefore, in environmental conditions where nutrient availability is very low (e.g. railways, city pavements, sandy beaches, etc.) a strong microbial N starvation could be expected, substantially limiting CBs decomposition. Differently, in presence of N source in the surrounding environment (e.g. underlying soil, sediments, N-rich water, etc.), microbial decomposers could acquire additional N from external sources, transfer it through fungal mycelia network into the N-poor organic material and so meet their nutritional requirements. Once the starvation constraints are overcome, the decomposition process proceeds at higher rates.

Aside from their environmental persistence, CBs also pose a threat to biological organisms living in terrestrial and aquatic ecosystems. Several authors reported that smoked CBs are toxic to polychaete (Wright et al., 2015), snails (Booth et al., 2015), mussel (Montalvão et al., 2019a), insects (Dieng et al., 2011), fishes (Slaughter et al. 2011), higher plants (Green et al., 2019), and also can interfere with bird behaviour (Suárez-Rodríguez et al., 2013). Recently, CBs were classified as hazardous according to the European regulation based on a battery of toxicity and ecotoxicity tests (Rebischung et al., 2018). Previous studies highlighted a greater toxicity of smoked versus unsmoked CBs (Novotny et al., 2011; Torkashvand et al., 2019) because the filter retains nicotine and many other compounds produced during tobacco combustion such as acetaldehyde, ammonia, benzene, formaldehyde, hydrogen cyanide, pyridines, and phenols (Hoffmann and Ilse, 1997). However, no studies investigated whether and how such toxic effects differ between undecomposed and decomposed smoked CBs. Indeed, whether the biological effects of CBs vary over time during decomposition as a results of the associated chemical changes is still an open question, limiting the knowledge about their environmental impact.

Data about CBs decomposition is also limited by the short-term duration of previous experiments (Joly and Coulis, 2018). To fill these gaps, we set up a long-term (5 years) experiment to assess the decomposition dynamics, including chemical and microbiome changes, and ecotoxicity of cellulose acetate CBs incubated in both controlled and field conditions considering two soil types (i.e. with contrasting physical-chemical properties), and with the absence of soil. CBs ecotoxicity was evaluated with multi-species bioassays using the bacteria *Aliivibrio fischeri* and the algae *Raphidocelis subcapitata*. Changes in organic carbon chemistry were identified by combining carbon-13 cross-polarization magic-angle-spinning nuclear magnetic resonance (^{13}C CPMAS NMR) spectroscopy with liquid chromatography-mass spectrometry (LC-MS). Moreover, CBs physical structure was visualized by scanning electron microscope (SEM). To describe the microbiome associated with CBs decomposition we used high-throughput sequencing of bacterial and eukaryotic rRNA gene markers. Specific aims were to: (i) describe CBs breakdown dynamics at long-term; (ii) verify the existence of N transfer from soil to CBs and evaluate its promoting role on the degradation process; (iii) assess whether and when CBs carbon chemistry and acetylation degree change during decomposition; (iv) describe the compositional dynamics of the CBs-associated microbiome; (v) investigate the changes in CBs toxicity occurring during the decay process and identify possible underlying chemical determinants.

2. Materials and methods

2.1. CBs preparation

Cigarettes of four common brands (regular filtered type) were purchased and artificially smoked (for details about the method see Bonanomi et al., 2015). A total of 12,000 CBs were used for the experiment. Smoked CBs of the four brands showed the following chemical composition (N = 10 for each brand): N content = 0.21 ± 0.12%; C/N ratio = 192.39 ± 12.11.

2.2. Decomposition experiment

Plant litter and organic matter decomposition in field conditions depends on water availability, temperature, N availability and organic matter chemistry. Here, CBs was decomposed both in laboratory and field conditions: in the first case the experiment was carried out to exclude the role of climatic conditions (temperature and soil moisture) and to isolate the interactive effects of CB chemistry and soil type on decay rate.

The litterbag method (Berg and McClaugherty, 2008) was used both in laboratory and field conditions. CBs were cut by scissors and pieces of 200 mg each were obtained. Litterbags were filled with 30 CBs pieces and then incubated in five environmental conditions: i. laboratory without soil (thereafter Lab, no soil); ii. laboratory in trays filled with park grassland soil (Lab, grassland soil); iii. laboratory in trays filled with sand dune soil (Lab, sand dune soil); iv. park grassland field (Field, grassland soil), and; v. sand dune field (Field, sand dune soil). CBs are commonly discarded in sand dunes and park grassland and for this reason such environments were selected for the experiment. The characteristics of vegetation types and soils of the park grassland and the sand dune are reported in Tables S1 and S2. The study sites are both located in the Regione Campania, Southern Italy (park grassland in Portici 40° 48′ 43″ N – 14° 20′ 49″ E; sand dune in Agropoli 40°25′10.11″N 14°59′13.34″).

In laboratory, the litterbags were placed in a chamber with controlled temperature (25 ± 2 °C at day and 22 ± 2 °C during the

night) and soil moisture (distilled water was added every seven days). Large trays (100 cm × 100 cm x 30 cm) filled either with sand dune or grassland soil were used to host the litterbags that were placed over the soil surface. The experimental design comprised five treatments (i.e. the environmental conditions where CBs were incubated), replicated with ten litterbags for all treatments and for each of the eight decomposition time planned i.e. 30, 90, 180, 360, 720, 1080, and 1800 days. After collection, the CBs were dried in a ventilated chamber at 40 °C until constant weight was reached, cleaned from soil debris and thereafter weighed to the nearest 0.001 g.

GLM was used to test main and second order interactive effects of incubation conditions (laboratory and field), soil type, and sampling date (treated as a continuous covariate) on CBs mass loss. Differences were statistically evaluated by post-hoc Duncan's test and significance evaluated at $P < 0.05$ and $P < 0.01$.

2.3. Chemical analyses

Total C and N content of CBs undecomposed and incubated for 30, 180, 720 and 1800 days were determined by flash combustion of microsamples (5 mg each) using an Elemental Analyser NA 1500 (Carlo Erba Strumentazione, Milan, Italy).

CBs (undecomposed and incubated for 720, 1080 and 1800 days) were analysed by ^{13}C-CPMAS NMR using a spectrometer Bruker AV-300 equipped with a 4 mm wide-bore MAS probe (details reported in Bonanomi et al., 2015). Spectral regions and corresponding C types were identified as follows: 0–45 ppm = alkyl C; 46–60 ppm = methoxyl and N-alkyl C; 61–90 ppm = *O*-alkyl C; 91–110 ppm = di-*O*-alkyl C; 111–140 ppm = H- and C- substituted aromatic C; 141–160 ppm *O*-substituted aromatic C (phenolic and *O*-aryl C); 161–190 ppm carboxyl C. The degree of acetylation of CBs was assessed by dividing the integral of the methyl C signal and the C 1 signal.

2.4. LC-MS analysis

CBs were extracted overnight, using a solution of water (H_2O) – methanol (MeOH) 80:20. The extract was first filtrated with Whatman filter paper and then using syringe filters with a 0.22 μm membrane. The analysis of CBs molecules was performed by a high-performance liquid chromatography (HPLC) 1260 Infinity Series (Agilent Technologies, Santa Clara, CA, USA). The system was coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer model G6540B (Agilent Technologies) with a Dual Electrospray Ionization (ESI) source that was equipped with a diode array detector (DAD) system (Agilent Technologies). A method was developed using a Titan C18 column (1.9 μm, 50 mm × 2.1 mm i.d., Supelco©, Bellefonte, PA, USA) for separations, held at constant temperature of 37 °C. The elution was performed injecting a sample volume of 10 μL and using a linear gradient composed by A: 0.002% (v/v) formic acid (FA) in H_2O and B: 0.002% (v/v) FA in MeOH with a flow rate set at 0.2 mL/min. The gradient was as follows: starting condition 0% B, held for 2.5 min, ramping to 100% B in 6.5 min, held at 100% B for 1 min, lowering to 0% B in 1 min and equilibration at 0% B for 2 min. DAD detector has recorded UV spectra every 0.4s from 190 to 750 nm with a resolution of 2 nm. The mass spectrometer operated in positive mode ($[M+H]^+$ ions) and MS spectra were recorded in centroid mode, with an *m/z* 50-1600 mass range and speed of 3 spectra/s. Capillary voltage was set at 2000 V, fragmentor at 180 V, cone 1 (skimmer 1) at 45 V, Oct RFV at 750 V. Drying gas flow was set at 11L/min at a temperature of 350 °C, and the nebulizer was set at 45 psig. An Isocratic pump (1260 Infinity Series, Agilent Technologies) was used to infuse a standard solution (to perform real-time lock mass correction), consisting of two reference mass compounds: purine ($C_5H_4N_4$, *m/z* 121.050873, 10 μmol/L) and hexakis (1H,1H,3H-tetrafluoropentoxy)-phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$, *m/z* 922.009798, 2 μmol/L). Flow rate was set at 0.06 mL/min, while the detection window and the minimum height were set at 1000 ppm and 10000 counts, respectively, for reference mass correction. All instrumental parameters were set with the Agilent MassHunter Data Acquisition Software, rev. B.05.01.

Statistical analysis of raw data was carried out by one-way ANOVA (p-value<0.05), using Mass Profiler Professional software (Agilent Technologies rev. B.13.00). A homemade plant metabolites database was consulted to identify compounds. Such data were subjected to further analysis.

2.5. CBs microbiota

Microbial DNA was extracted using DNeasy PowerSoil kit (Qiagen). Bacterial diversity was studied by sequencing of the amplified V3-V4 region of the 16S rRNA (Klindworth et al., 2013), while primers BITS2F/B58S3 were used for the fungal community analysis (Bokulich and Mills, 2013). Library preparation and sequencing was carried out as described by Berni Canani et al. (2017). The amplicons were combined in two separate equimolar pools and sequenced on an Illumina MiSeq platform, leading to 2 × 250 bp reads.

2.6. Bioinformatics data analysis

After demultiplexing, Illumina adaptors were removed using cutadapt (https://cutadapt.readthedocs.io/en/stable/). FLASh (Magoč and Salzberg, 2011) and PRINSEQ (Schmieder and Edwards, 2011) were used to join paired-end reads and quality filtering, respectively. Reads were trimmed at the first base with a Phred score lower than 20: reads shorter than 300 bp (Bacteria) or 100 bp (Fungi) were discarded. Data were analysed by QIIME 1.9.1 software (Caporaso et al., 2010). Abundances were normalized at the lowest number of sequence/samples to avoid biases due to the different sequencing depth. OTU tables were imported in R environment for further analyses and visualization (http://www.r-project.org). Sequence data were made available in the Sequence Read Archive of the National Centre of Biotechnology Information (accession number Bioproject PRJNA526990).

In order to assess significant variation in fungal and bacterial communities we perform Permutation analysis of variance (PERMANOVA). Specifically, we tested the effect of environmental conditions considered it as fixed factor, with soil types and decomposition times considered as nested in environmental decomposition and as random factors. PERMANOVA was carried out in Primer 7 software following unrestricted permutation methods (999 Number of permutations).

2.7. Ecotoxicological assay

The ecotoxicity of CBs was determined by preparing leachates according to UNI EN 12457-2:2004 modified (i.e. 24 h contact time mixing at 10 rpm with artificial freshwater (AFW) (ISO 2012 as leaching solvent and a solid-to-liquid ratio 1:100 (1 g cig butt/100 mL of AFW). Toxicity tests were carried out with *Aliivibrio fischeri* (bacterium) and *Raphidocelis subcapitata* (microalgae) considering at least five serial leachates' dilutions (v/v). The ISO (2007) method was used for *A. fischeri* (NRRL B-11,177) considering as endpoint its bioluminescence inhibition via the Microtox® luminometer (Microtox Model 500; Microbics Corp., USA). The algal growth inhibition assay with *R. subcapitata* was carried following ISO (2012). Toxicity was expressed as percentage of effect (PE) or median effect concentration (EC50). When EC50 were

available, data were expressed as toxic units (TU50 = 100/EC50) (Libralato et al., 2010).

GLM was used to test effects of incubation conditions (laboratory and field), soil type, and sampling date (treated as a continuous covariate) on CBs ecotoxicity. Two GLMs were separately fitted for *R. subcapitata* and *V. fischeri* bioassays. Differences were statistically evaluated by post-hoc Duncan's test and significance evaluated at $P < 0.05$ and $P < 0.01$.

2.8. Scanning electron microscopy

To investigate CBs morphology, samples (5 × 5 mm) were dehydrated with ethanol (increasing concentration) under a laminar flow cabinet. Subsequently, the samples were dried with CO_2 in a critical-point dryer EMITECH K850 (Emitech, Ashford, UK). Samples were then mounted in aluminum stubs and finally coated with gold/palladium in a sputter coater (AGAR B 7340, Agar Scientific Ltd, Stansted, UK). Specimens were examined with scanning electron microscope Zeiss EVO40 at 20.00 KV EHT, at 11.5 mm < WD < 16.5 mm and at several magnifications (from 1 K to 2.4 K).

3. Results

3.1. Mass loss

CBs mass loss was significantly affected by the incubation conditions, soil type and decomposition time, with generalized linear model (GLM) that highlighted significant first-order and interactive effects (Table S3). CBs mass loss followed a three-steps decomposition pattern. A rapid mass loss consisting of ~15–20% of initial mass was verified in the first month of incubation and was associated with the rapid degradation of the external cellulose layer (Fig. 1A and Fig. S2). In the following two years, however, decomposition was very slow reaching a total mass loss of ~30–35% (Fig. 1A). Starting from the third year of incubation, a treatment-dependent mass loss clearly emerged, with significant differences compared to the first two decomposition phases and consistent until the fifth year of observation (Supplementary Table S4). CBs incubated in the laboratory condition showed a large mass loss over both soil types, reaching 75% and 81% after 5 years for grassland and sand dune in laboratory, respectively, much higher than samples incubated without soil (52%). CBs decomposing in field conditions showed intermediate mass loss values, as related to the enhancing effect of soil addition coupled with sub-optimal environmental conditions. SEM images showed that, in absence of soil, the physical structure of CBs filters remained almost unchanged even after 5 years, with cellulose acetate fibres still well conserved (Fig. 1B and Fig. S3). Such physical structure still held in CBs decomposing in presence of soil in laboratory condition, as well as in the field, up to 2 years of incubation. Differently, after 5 years of incubation the CBs fibres were largely degraded, appearing both bitten and scratched, as in the case of those incubated in field condition in sand dune (Fig. S3). Noteworthy, CBs appeared completely dissolved into an amorphous organic matrix when decomposed over the park grassland soil in laboratory conditions (Fig. S3).

3.2. Nitrogen transfer

Consistently across treatments, N concentration and total N content in CBs did not change in the first six months of incubation. Thereafter, however, N concentration and content increases after two years in samples incubated in field condition in park grassland as well as in those decomposed in the sand dune (Fig. 2). Such pattern was consistent with a significant N transfer from the soil compartment to CBs, especially in grassland field. After 5 years of incubation, N concentration even increased in grassland CBs, while a slight decrease of total N content in both field conditions indicated a N loss by mineralization. It is noteworthy that in absence of soil in laboratory condition, total N content did not vary substantially over the experiment duration. The C/N ratio decreased during decomposition, reaching lower values in case of CBs incubated in the grassland field (Fig. 2).

3.3. Chemical changes assessed by ^{13}C CPMAS NMR and LC-MS

Pure cellulose showed typical glucose peaks at δ 62-63 (C-6), δ 69-76 (C-2/C-3/C-4/C-5) and δ 105-106 (C-1) (Fig. S1). In addition to these, ^{13}C CPMAS NMR spectra of smoked, undecomposed CBs also showed two major peaks at δ 19-21 and δ 170-175, associated to the signals of methyl and carboxyl carbons of the acetyl groups, hence reflecting the degree of CBs cellulose acetylation. After 2 years of decomposition, CBs showed at most slight chemical changes (Fig. 3), with minor decrease in the degree of cellulose acetylation (from 2.38 to 2.32 for undecomposed and decomposed CBs, respectively). The ^{13}C NMR spectra, and specifically the signals diagnostic of cellulose acetate, showed a consistent pattern, with peaks substantially unvaried, consistently across treatments. Differently, the same signals showed a sharp change after 3 and 5 years of incubation. Specifically, the reduction of signal intensity at δ 19-21 and δ 170-175 indicated the loss of the acetate group from cellulose acetate, likely as result of ester bond breaking. CBs deacetylation was observed in the spectra of samples decomposed in laboratory conditions, also in absence of soil, while was less evident in field conditions (Fig. 3). Interestingly, the observed deacetylation was paired to a degradation of the most dominant *O*-alkyl C fraction in all treatments, but not in the case of CBs incubated in laboratory in absence of soil, where instead it showed an increase in its relative abundance (Fig. 3). This result likely reflects the limited mass loss and the lack of fiber degradation observed in absence of soil. On the contrary, in treatments with grassland soil in laboratory, a sharp decrease of the *O*-alkyl C fraction indicated the degradation of the cellulose fibers. Moreover, in these samples the aliphatic alkyl-C region (characteristic of lipid cutins, waxes, and microbial by-products) increased sharply after 3 and especially 5 years (Fig. 3), likely related to the chemical nature of the amorphous physical structure highlighted above (Fig. S3). A smaller but significant increase of the aliphatic fraction was also observed in CBs decomposed in field conditions and in laboratory with the sand dune soil. A decrease in the aromatic fractions (δ 111-140 and δ 141-160) were recorded after 2 years in all incubation conditions (Fig. 3). These variations likely reflect the degradation of the numerous compounds of phenolic and aromatic origin present in smoked CBs. Accordingly, LC-MS data consistently showed the sudden disappearance of 31 putative compounds exclusively occurring in undecomposed CBs (Fig. S5). After 5 years of decomposition, however, the relative abundance of the aromatic fractions slightly increased, suggesting a possible formation of new compounds (Fig. 3).

3.4. Microbiota

Proteobacteria dominated CBs microbiome, although their levels decreased during CBs decomposition, with this phylum being progressively replaced mostly by Firmicutes in samples incubated in laboratory conditions, and by Actinobacteria in grassland and sand dune fields (Fig. S4A). Non-metric multidimensional scaling (NMDS) based on microbial community composition showed a clear separation of the samples according to incubation conditions

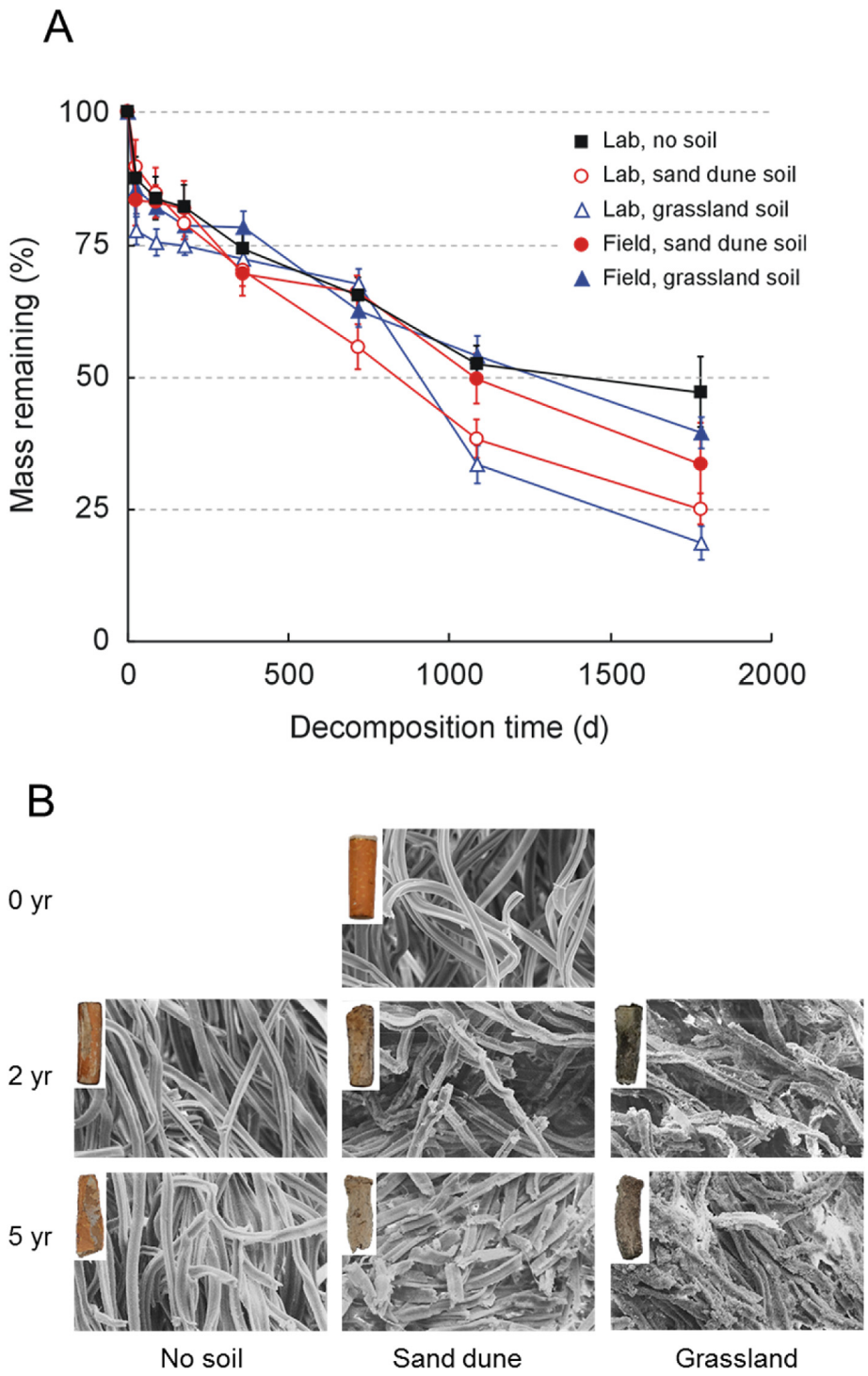

Fig. 1. CBs decomposition during a 5 years incubation period. (A) Cigarette butts mass remaining during a 1800-day decomposition period in laboratory and field conditions. Data refer to mean ± standard deviation of 10 replicates for each material (statistical details in Tables S3 and S4), (B) SEM images of undecomposed, 2 and 5 years old CBs, insets shows not magnified pictures of CBs at the corresponding decomposition stage.

(field *vs.* laboratory) and CBs age (Fig. 4A). In addition, field samples showed higher levels of time- and soil-dependent diversity (Fig. 4A). PERMNANOVA showed that bacterial and fungal communities were affected significantly by the experimental factors (Supplementary Tables S5 and S6). The most abundant genera (i.e. relative abundance >1% in at least one CBs sample), the majority showed an evident shift in the relative abundance during the decomposition process (Fig. 4B). *Massilia* and *Pseudomonas* were abundant at early decomposition stage, but were replaced by *Bacillus*, *Planctomycea*, and *Rhodospirillaceae* at the later phases. Remarkably, *Azospirillum* members were most abundant at the intermediate decomposition stage, but only into CBs incubated in laboratory conditions, mostly with the sand dune soil or without soil addition.

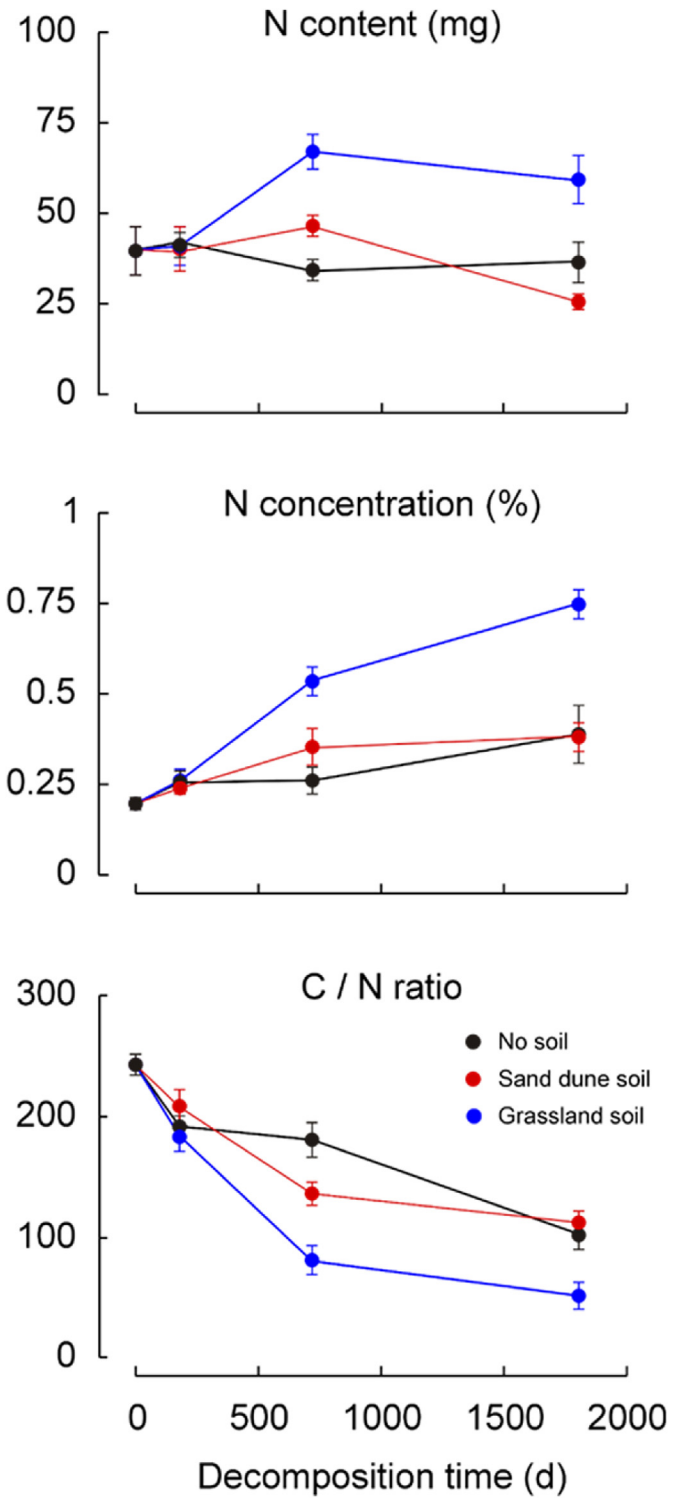

Fig. 2. Nitrogen dynamics within CBs during decomposition. Total nitrogen content (above), nitrogen concentration (middle) and C/N ratio (below) in cigarette butts during the 1800-day decomposition period. Data refer to field conditions for sand dune and grassland and to laboratory for no soil treatment. Values are mean ± standard deviation.

The fungal microbiota was dominated by Ascomycota and Basidiomycota (Fig. S4B). NMDS showed that fungal microbiota changed over decomposition time, with successional shifts more pronounced for CBs incubated in field conditions compared to samples decomposed in laboratory (Fig. 4C). Specifically, CBs incubated in the sand dune showed a substantial increase in the levels of Basidiomycota over time, which exceeded 80% of all Fungi at the late incubation stages (Fig. 4D and Fig. S4B). At genus level, members of *Amphinema*, *Lepiota*, *Pysolithus*, and *Rhizopogon* were exclusively found in CBs from the sand dune field, along with members of *Xylaria*, an Ascomycota showing specific capability to degrade recalcitrant substrates. CBs fungal community in laboratory conditions showed small changes over time, irrespective of soil presence and type, likely due to the lack of exogenous contributions from the preexisting fungal community of the surrounding environment (Fig. 4C). In such conditions, Ascomycota dominated the CBs fungal community. Successional changes were limited to Ascomycota, with members of *Aspergillus*, *Eurotium*, and *Sclerotinia* initially abundant and then largely replaced by *Phoma*, *Penicillium*, and *Paraconiothyrium* (Fig. 4D).

3.5. Ecotoxicity

CBs ecotoxicity was assessed with standard bioassays, using the alga *Raphidocelis subcapitata* and the gram-negative bacterium *Aliivibrio fisheri*. Both species underwent significant adverse effects when exposed to water extracts of smoked, undecomposed CBs (Fig. 5A and B). However, CBs toxicity started to decline after 30 days of decomposition, mirroring the disappearance of 31 putative compounds identified by LC-MS in the same extracts (Fig. S5). Consistently, nicotine degradation was very rapid, completely disappearing after 30 and 90 days in presence and absence of soil, respectively (Fig. 5C). After the early decomposition stage, CBs toxicity to *A. fischeri* levelled off, with minor differences among treatments, but still with high levels of toxicity (approximately 15–40 TU50), while in the case of *R. subcapitata* the inhibitory effect resurged during CBs decomposition, especially for treatments with sand dune soil in laboratory (Fig. 5B, Supplementary Table S7). Interestingly, LC-MS showed several cases of compounds, including different polyethylene glycol forms (i.e. PEG13, PEG14), with content increasing over CBs decomposition time (Fig.s 5D, S6). Out of these, a correlation analysis based on ecotoxicity and LC-MS data for all the sampling dates with the exclusion of undecomposed CBs, revealed that in 14 cases the compounds content in CBs extracts were positively associated with the magnitude of *R. subcapitata* inhibition exerted by the same extracts (Fig. 5E).

4. Discussion

Our long-term experiment revealed that CBs decomposition follows a three-stages process. The first stage, that lasts few weeks, is characterized by a rapid CBs mass loss independent by soil presence, type and incubation conditions. The observed mass loss of ~15–20% is likely associated to leaching of soluble compounds and degradation of the cellulose layer that wraps the filter. The complete disappearance of the wrapping layer, especially in field conditions (Fig. S2), support this hypothesis. Early decomposition could be associated to non-specialized microbiota, consistently with the NMDS results that showed minor differences among bacterial and fungal communities after one month. Within the same time frame, LC-MS results highlighted the sudden decay of several low-molecular weight compounds, including nicotine, for treatments encompassing soil addition and/or field conditions, while in absence of soil, nicotine depletion required more than 6 months. The rapid degradation of those low-molecular weight compounds was associated to the dynamics of CBs toxicity on bacteria and algae. According with previous studies (Slaughter et al., 2011; Green et al., 2019; Montalvão et al., 2019b), undecomposed, smoked CBs are highly toxic for a range of organisms

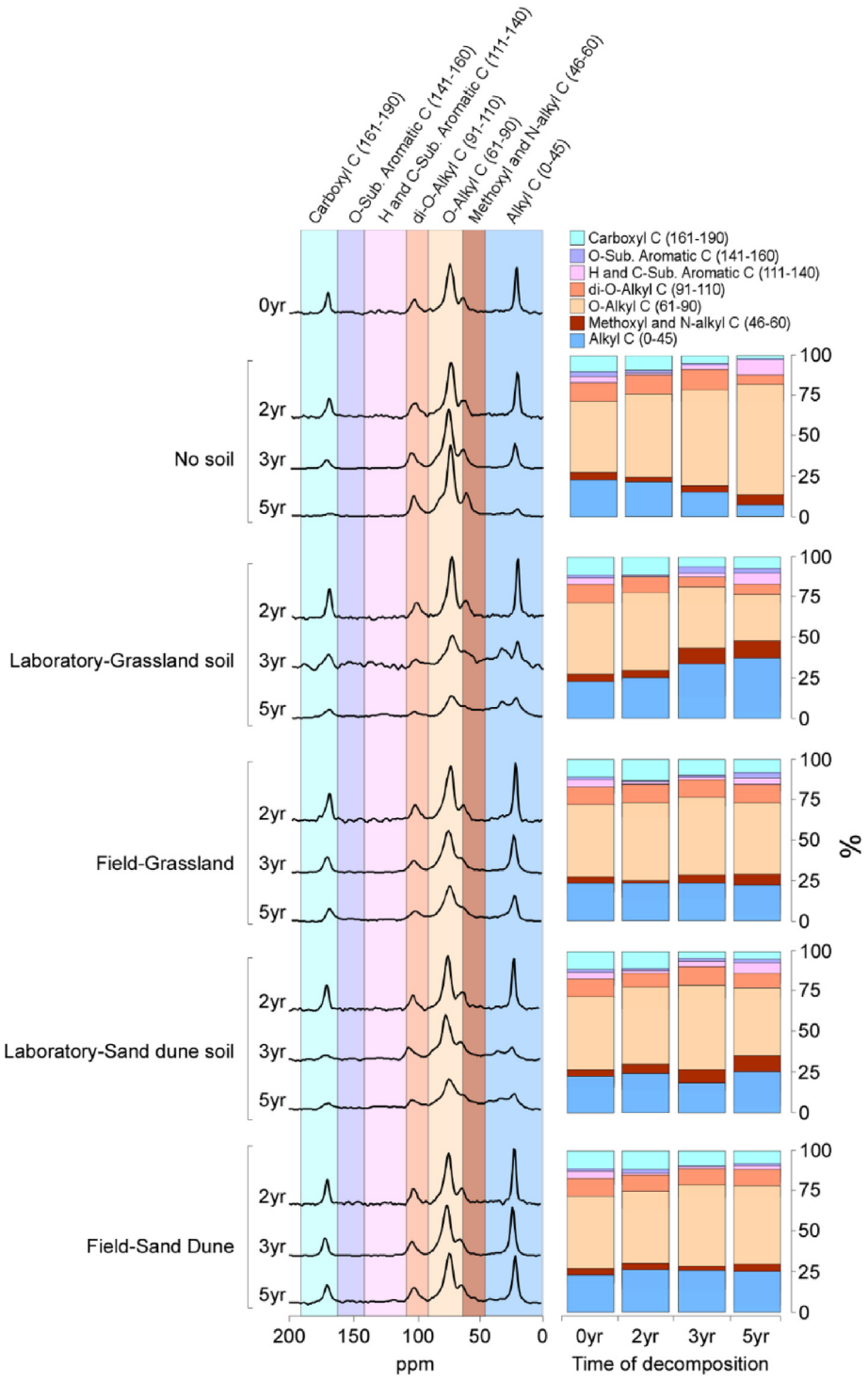

Fig. 3. CBs variation in carbon composition as assessed by ^{13}C CPMAS NMR spectroscopy. (A) Spectra of CBs either undecomposed or after 2, 3, and 5 years of decomposition in laboratory and field conditions and (B) related peak integrations in the seven spectral regions.

(review in Torkashvand et al., 2019). However, our results clearly demonstrate that CBs inhibitory effects abruptly drop down after just 30 days of decomposition, reflecting the rapid degradation of nicotine and several other compounds (Fig. 5 and Fig. S5). Recently, Selmar et al. (2018) reported that improper disposal of CBs in agricultural fields by farm workers results in a substantial contamination of vegetable production because nicotine uptake by plants. Substantial pollution was recorded with a single CB per square meter, with detectable alkaloid leaching into the soil and subsequent uptake by different crops.

During the second decomposition stage (~2 years), CBs decomposition proceeds slowly, with only a further ~10.0% mass loss from on month to two years. Such dynamics mirrors that observed for leaf litter decomposition, characterized by a rapid depletion of the labile C fraction, most contributed by soluble carbohydrates, and the preservation of the recalcitrant C fraction,

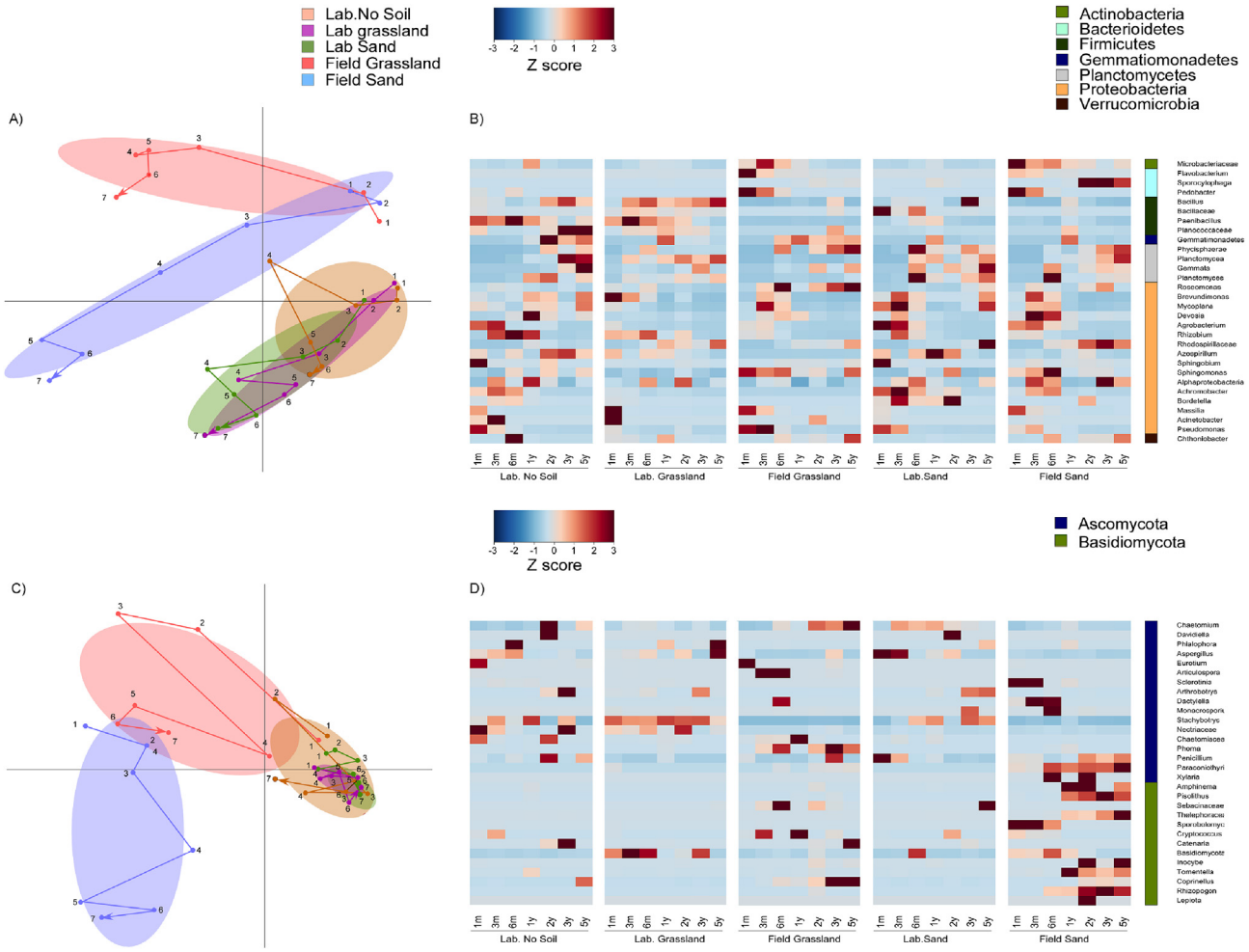

Fig. 4. Succession of bacterial and fungal communities over CBs. NMDS of CBs at seven decomposition stages (1 = 1 month; 2 = 3 months; 3 = 6 months; 4 = 1 year; 5 = 2 years; 6 = 3 years; 7 = 5 years) based on the relative abundance of bacterial (A) and fungi (C) phyla found in the samples. The relative abundance of most abundant genera is also reported for bacterial (B) and fungi (D). Different colors reported on the right-side of each heat-maps group indicate the phyla to which genera belong. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

including lignin and, for some leaf types, complex lipids of waxes and cutin (Kögel-Knabner, 2002; Berg and McClaugherty, 2008). Interestingly, during the second decomposition stage, CBs decay rate differences among treatments were substantially negligible in any of the conditions. CBs decomposition in this time frame could have been hampered by two main factors: the intrinsic resistance to biodegradation of cellulose acetate having a substitution degree of ~2.45, and the limited N availability into the CBs eventually leading to microbial starvation. In this respect, it is well established that soil microbiota capable of decomposing N-poor plant tissues (i.e. wood and coarse root), can actively attain inorganic N from the surrounding soil to sustain their biological requirements (Hodge et al., 2000). Noteworthy, dynamics of CBs N concentration, total N content, and C/N ratio, consistently showed an increase of N availability in CBs during the second stage of decomposition in treatments with soil addition, with higher magnitude for N-rich grassland soil compared to sand dune, but not in absence of soil. These data support the hypothesis of N transfer, mediated by the microbiota, from the soil to the CBs. N transfer among leaf litter decomposing in mixtures has been previously reported and related to three processes: passive diffusion, leaching, or the active transport through fungal mycelia networking (Lummer et al., 2012). Unexpectedly, at least considering previous evidence on plant litter mixtures (Bonanomi et al., 2017), in the case of CBs the net N transfer did not translate into increased mass loss rates. One plausible reason for such different behaviour could be related to a difference in the molecular composition of the recalcitrant C fraction between CBs and plant debris, i.e. lignin and complex lipids *vs.* plasticized cellulose acetate. Accordingly, for CBs aged 720 days we calculated a high substitution degree of 2.32. This value demonstrate that, after two years of decomposition, CBs was still poorly de-acetylated and hence hardly susceptible to microbial decomposition. These results suggest that, even in conditions of sufficient N availability, CBs should be de-acetylated to a low degree of substitution, prior to be efficiently processed by decomposers. Accordingly, after 2 years of incubation, we observed only minor changes in CB molecular composition and physical structure.

After three to five years of decomposition, all measures followed very different trajectories across treatments. CBs incubated in laboratory conditions, after the fifth year of observation, lost ~50% and ~80% of their initial mass in absence of soil and in presence of grassland soil, respectively, suggesting that five years of incubation under optimal conditions are sufficient for a complete de-acetylation of compressed fibres of cellulose acetate with a starting degree of substitution of ~2.45. Such observation in laboratory condition is surprising, considering that previous studies

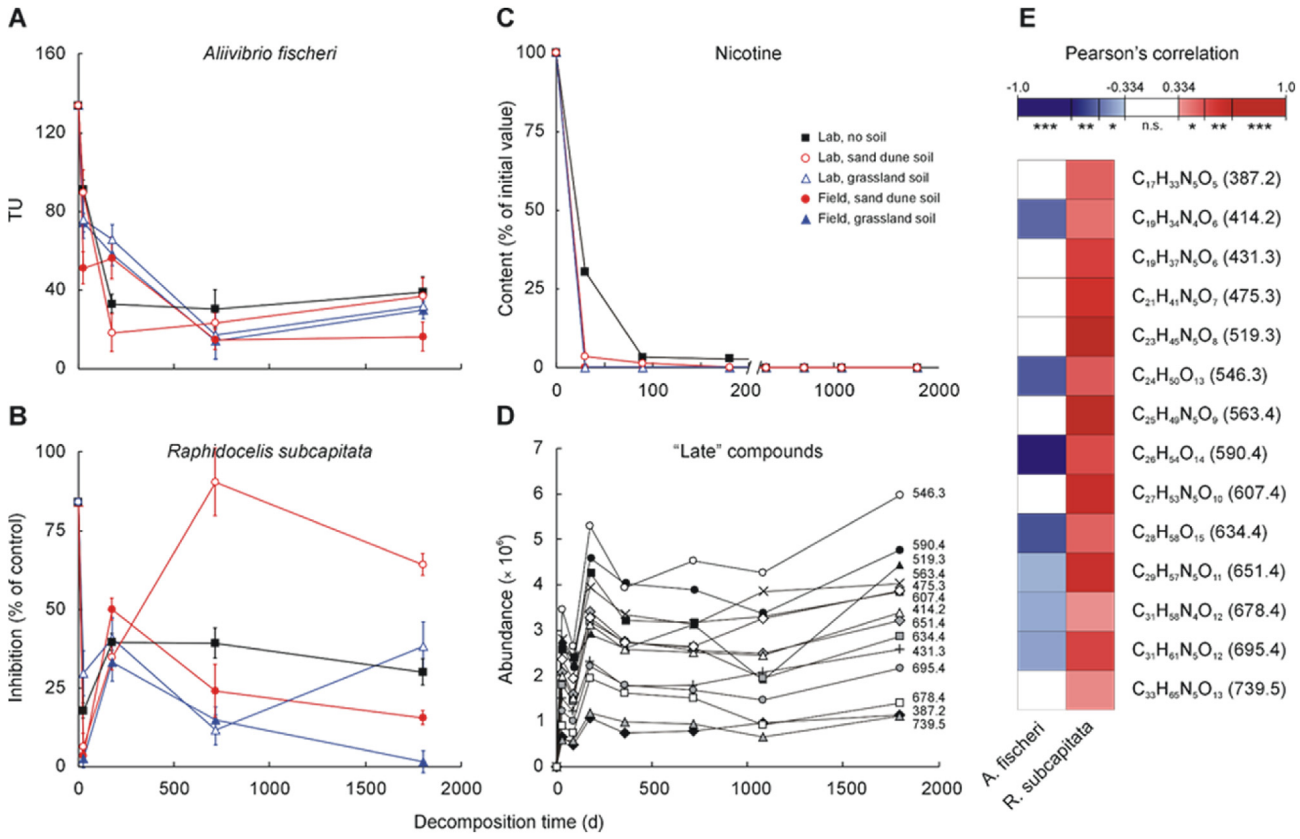

Fig. 5. Ecotoxicity of CBs and associated chemical compounds. Results of cigarette butt ecotoxicity assays (A, B), dynamics of nicotine (C) and content of 15 LC-MS detected compounds selected for their significant variability (D) over decomposition time and across treatments. Ecotoxicity for *Aliivibrio fischeri* is expressed as toxic units (TU). Heat map of correlation between cigarette butt ecotoxicity and chemicals (E). (A-B) Data refer to mean ± standard deviation of 10 replicates for each treatment and butt age (statistical support in Supplementary Table S6). Stars below the color bar in (E) specify statistical significance of Pearson's correlations scores (***: $p < 0.001$, **: $0.001 \leq p < 0.01$, *: $0.01 \leq p < 0.05$, n.s.: not significant). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

highlighted a central role of UV-radiation in CBs de-acetylation (Puls et al., 2011). Our study demonstrates that de-acetylation can occur also in absence of UV-radiation, although the underlying mechanisms remain unknown, and a role of the microbiota in such dynamics can be hypothesised. Despite the complete cellulose de-acetylation observed after five years of incubation, CBs fibrous structure underwent at most minor degradation, as supported by SEM and ^{13}C NMR results. Indeed, microbial degradation of the cellulose fibres would have produced a reduction of the *O*-alkyl C fraction associated to carbohydrates. Contrarily, this fraction showed an increase resulting from the reduction of the carboxyl and alkyl C fractions by de-acetylation. All these evidences consistently indicate that the cellulose in CB fibres, even after five years of incubation, was not exploited as a suitable substrate by microbes, despite its low degree of acetylation. We hypothesise that, in absence of soil or other external N sources, the limited N availability did not allow the progression of decomposition.

Different from CBs incubated in absence of soil, those decomposing over N-rich grassland soil in laboratory conditions showed the largest chemical and structural changes throughout the final decay stage. The progressive reduction of the *O*-alkyl C fraction, concomitant with the relative increase in the alkyl C fraction, were a further demonstration of cellulose degradation and accumulation of microbial biomass and necromass (Kögel-Knabner, 2002). This pattern resembles the changes in C quality observed in decomposing leaf litter following microbial substrate consumption (Preston et al., 2009). We speculate that, in presence of exogenous N sources, microbes were able to transfer N to CBs during the preceding stage. Then, after cellulose acetylation reached a sufficiently low substitution degree, saprotrophic microbes became responsive to the reduced C/N ratio of the substrate, proceeding with cellulose fibres degradation. This is consistent with the CBs mass loss observed after five years of incubation (~80% of CBs initial mass), their N content dynamics, and the molecular composition, rich in aliphatic C compounds, and the amorphous physical structure of the CBs residues. Considering ecotoxicity dynamics, CBs inhibitory effects towards the alga *R. subcapitata* showed a bimodal dynamic, with a notable resurgence following the initial peak and the subsequent abrupt decrease observed at the preceding stages. Several putative compounds were significantly associated to this pattern, including plastic derivate compounds with short C chains (i.e. PEG13, PEG14) and pyridine derivatives. These compounds, or at least some of them, may be produced in the early decomposition phase (3–6 months) and thereafter, by resisting to microbial degradation, progressively increase in concentration because of the ongoing CBs mass loss (Fig. 5). Such hypothesis is consistent with the observation that toxicity towards *R. subcapitata* at the late decomposition stage is highest for the CBs with the highest mass loss rates, while that of *A. fischeri* did not further reduce below 15-40 TU50. In this respect, data from the next sampling dates (the final sampling date of our experiment is planned after 10 years of incubation, in 2023) will shed further light on the fate and properties of the organic fraction of decomposed CBs residues.

CBs decomposition trajectories for all the remaining treatments followed a pattern like that described above, except for samples incubated in sand dune field. In such conditions, despite cellulose

fibers underwent a limited de-acetylation, CBs after 5 years lost ~66% of their initial mass. SEM micrographs highlighted a remarkable structural alteration of CBs, with signs of stretches, bites and fragmentation occurred during the incubation process. However, the activity of rodents or large arthropods must be excluded because of the small mesh size (1 mm) of the litterbag. On the other hand, CBs decomposing in sand dune hosted a specific, well differentiate and unique microbiota characterized by genera of fungi capable to degrade recalcitrant substrates such as *Lepiota*, *Pysolithus*, *Rhizopogon*, and *Xylaria*. Apparently, the trophic activity of these fungi may have determined a completely different trajectory to CBs decomposition that, in these conditions, could proceed irrespective of the cellulose de-acetylation process. Accordingly, the enzymatic activities of these fungi may have contributed to the observed fragmentation of cellulose acetate fibers.

5. Conclusions

According with previous studies (Novotny et al., 2011; Torkashvand et al., 2019), CBs are most toxic immediately after smoking, with inhibitory effects rapidly decreasing during decomposition. Noteworthy, our study revealed a second toxicity peak emerging at intermediate-to-late stage (two to five years), clearly indicating a long-term hazard posed by CBs discarded in the environment. Future research should be directed to identify the chemical determinants of CBs ecotoxicity, as well as and longer-term impacts on terrestrial and aquatic ecosystems. Our study, moreover, revealed that CBs decompose very slowly over the first two years but, thereafter, take different trajectories in relation to the presence of exogenous N sources and local microbiome composition. In absence of soil, a common situation in urban environment, CBs decay rates are mostly limited by microbial N starvation. Differently, in presence of soil, a net N transfer in CBs from the surrounding environment promotes the decomposition process. However, N promotes CBs decomposition only when cellulose acetylation falls below a threshold of substitution degree, so that microbes can efficiently use N and convert CBs fibers into an amorphous, aliphatic-rich organic material. Remarkably, in sand dune where the soil microbiome is dominated by several Basidiomycota species, capable to feed upon recalcitrant organic C, CBs decomposition can proceed irrespective of cellulose de-acetylation. Finally, our findings highlight the importance of long-term studies to reveal the fate and impact of CBs in different environments.

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Novelty statment

Cigarette butts (CBs), by number, are the most common littered item on Earth. CBs pose a serious threat to living organisms and ecosystem health when discarded in the environment because they are toxic to microbes, insects, snails, fish, birds, and mammals. Recently, CBs was classified as hazardous waste according to the European regulation.

Despite CBs persistence and toxic hazard, no long-term studies have addressed the effects of environmental conditions on CBs decomposition rate and ecotoxicity. Here, for the first time, our long-term (5 years) decomposition experiment clarify the fate of CBs in realistic ecological conditions and highlight their ecotoxicological impact.

Author contribution

G.B. conceived the study. G.B., G.C., Z.M. realized the decomposition experiment. G.M., A.D. made nitrogen analysis. P.M., A.P made and analysed ^{13}C-CPMAS NMR data. A.S., F.V. made and analysed LC-MS data. A.C made SEM pictures. F.D, A.L. D.E. carried-out the microbiome analyses. A.S., G.L., M.G. made the ecotoxicological analyses. G.B. and G.I. write the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114108.

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