



Cigarette butts, a threat for marine environments: Lessons from benthic foraminifera (Protista)

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ABSTRACT

Cigarette butts are the most common form of litter in the world and their environmental impact is related to both persistence and potential toxic effects for chemical composition. The objective of this study was to assess the acute toxicity (LC₅₀-48 h) of human-smoked cigarette butts leachate on 3 cultured genera of benthic foraminifera: the calcareous perforate *Rosalina globularis*, the calcareous imperforate *Quinqueloculina* spp., and the agglutinated *Textularia agglutinans*. The specimens were exposed to 16, 8, 4, 2, and 1 cigarette butts/L concentrations that prove to be acutely toxic to all taxa. Starting from 4 cigarette butts/L, both calcareous genera showed shell decalcification, and death of almost all the individuals, except for the more resistant agglutinated species. These results suggest the potential harmfulness of cigarette butts leachate related to pH reduction and release of toxic substances, in particular nicotine, which leads to physiology alteration and in many cases cellular death.

1. Introduction

Cigarette butts are one of the most common forms of litter in the world, as approximately 5.6 trillion cigarettes are smoked every year worldwide (Parker and Rayburn, 2017). Cigarette waste constitutes an estimated 30% of the total litter (by count) on US shorelines, waterways, and on land (Litter Free Planet, 2009). About 21% of the total litter has been estimated across Europe's beaches (The European Environmental Agency, 2014-2017). Although their impact on human health has been widely described in the literature (Saha et al., 2007), the possible ecological impact caused by the indiscriminate cigarette butt disposal remains poorly known. According to Williams et al. (2016) and Cardoso et al. (2018), cigarette butts can be dispersed into watercourses, lakes, seas, and soils during the rainy season; the environmental impact of cigarette butt waste is related to their persistence in the environment,

potential toxic effects (Lee, 2012), and their chemical composition. A large number of chemical products are used in the growth and processing of tobacco and the manufacture of cigarettes, including fungicides, herbicides, insecticides, and pesticides (Glantz and Forbes, 1996). Reports (Li et al., 2002; Hoffmann et al., 1997) have shown that cigarette particulate matter (tar) and mainstream smoke (ms) contain more than 4000 chemicals, including hydrogen cyanide, nitrates, ammonia, acetaldehyde, formaldehyde, benzene, phenol, pyridines, and carbon monoxide. More than 50 of these are known human carcinogens (Hoffmann et al., 1997). Among them, attention has been dedicated to the occurrence of nicotine that represents a toxic alkaloid largely consumed in the world as a constituent of tobacco smoke (van Wel et al., 2016). This substance is found mainly in the water compartment (93%, Canadian Environmental Modeling Center level III model) where, due to its chemical features, can be easily bioavailable to organisms and

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produce toxicity (Oropesa et al., 2017; Robles Molina et al., 2014; US EPA, 2009; Hansch et al., 1995). Moreover, previous studies have shown that chemicals in cigarette butt leachate can be acutely toxic to aquatic organisms including microorganisms (*Vibrio fischeri*; Micevska et al., 2006), zooplankton (*Daphnia magna* and *Ceriodaphnia dubia*; Micevska et al., 2006; Register, 2000; respectively), tidepool snails (three species: *Austrocochlea porcata*, *Nerita atramentosa* and *Bembicium nanum*; Booth et al., 2015), frogs (*Xenopus laevis* larvae; Parker and Rayburn, 2017), ragworm *Hediste diversicolor* (Wright et al., 2015), and amphipod species *Grandidierella japonica* (Hiki et al., 2018). Among fishes, we report the freshwater flathead minnows, *Pimephales promelas*, the marine topmelt, *Atherinops affinis* (Slaughter et al., 2011), and embryos of the Japanese rice fish *Oryzias latipes* (Lee and Lee, 2015). Acute toxicity occurs at levels as low as 1 cigarette butt per litre of water. Most of these ecotoxicological studies focus on non-vertebrate species (prokaryotes as marine bacteria and metazoan as daphnids) or fishes, where experimental investigations using protists to test the toxicity of cigarette butts in water are still missing. In particular, this study is dedicated to foraminifera (protists) because studies on this phylum investigating the toxicity of cigarette butts have not been performed yet. Worldwide-occurring foraminifera are known to respond to pollution through changes in assemblage makeup and abundance (e.g., Alve, 1995; Emrich et al., 2017; Yanko-Hombach et al., 2017), and to display a variety of morphological abnormalities that are considered a biological response to environmental stresses (e.g., Samir and El-Din, 2001; Yanko et al., 1998). In particular, benthic foraminifera also react to change in abiotic and biotic factors (Alve, 1995; Murray, 2006), and are often among the last eukaryotic organisms to disappear completely from polluted sites, making them exceptional bioindicators (Alve, 1991; Schafer, 2000). In fact, the assemblages constitute the most diverse and widespread group of shelled microorganisms in modern oceans (Sen Gupta, 1999), have relatively short life-cycles, and the potential to respond faster to changes in environmental conditions (e.g. Alve, 1995; Debenay et al., 1996; Jorissen et al., 2009; Frontalini and Coccioni, 2011; Bouchet et al., 2012). The study of pollution effects on benthic foraminifera and their use as proxies started in the 1960s (Resig, 1960; Watkins, 1961; Boltovskoy, 1965), and has increasingly developed in recent decades through environmental research (e.g., Alve, 1991; Alve, 1995; Yanko et al., 1999; Coccioni, 2003; Geslin et al., 2000; Geslin et al., 2002; Debenay et al., 2001; Debenay et al., 2005; Coccioni et al., 2005; Frontalini and Coccioni, 2008; Polovodova and Schönfeld, 2008; Armynot du Châtelet and Debenay, 2010; Martins et al., 2015). Despite these, there is a lack of information about quantitative pollutant-specific responses of foraminifera. Assessing the role played by a single pollutant, in the field, is a difficult task and laboratory experiments, through which benthic foraminiferal response to various types and concentrations of pollutants can be observed through time, represent the most effective and direct method to assess the effect of a single parameter (i.e., pollutant) on the benthic foraminiferal assemblages. Unfortunately, only a limited number of experiments have been conducted so far (i.e., Ernst et al., 2006; Frontalini and Coccioni, 2012; Frontalini et al., 2015, 2018).

Therefore, the aim of this paper is to investigate the acute toxicity (LC₅₀-48 h) of cigarette butt leachate on three selected genera of benthic foraminifera and the possible effects. This is a first attempt to understand the reaction of this toxicity in a group of unicellular eukaryotic organisms that forms part of a key link in marine trophic chains.

2. Materials and Methods

2.1. Foraminiferal cultures

Foraminifera, used in this study, were isolated from sediment samples collected by Van Veen grab from the central Adriatic and Mediterranean Sea Italian coastal areas. Specimens of *Rosalina globularis*, *Quinqueloculina* spp. and *Textularia agglutinans* were grown in isolated

monospecific cultures set up at the Laboratory of Paleocology at the DISVA (UNIVPM) and maintained since several years (Fig. 1). *Rosalina globularis* is a perforate calcareous species which precipitates CaCO₃ to build its test; their shells are usually made of low Mg calcite and this group is perforated with numerous microscopic pores that are found on most of the surface area of the shells (Erez, 2003). In addition, *R. globularis* is a common symbiont foraminiferal species routinely used as a model in ecotoxicological studies (Schintu et al., 2015). *Quinqueloculina* spp., including *Quinqueloculina seminula* and *Quinqueloculina* sp. are imperforate (miliolids/porcelaneous) foraminifera; miliolids precipitate their needle-shaped calcite crystals (usually high Mg calcite) within intracellular vacuoles. *Textularia agglutinans* is an agglutinated foraminifera that allocates particles of different type and size in a sufficient quantity to create a new case gluing them with a biomineralized calcium carbonate cement (Sen Gupta, 1999), despite there are studies showing that not all agglutinated species have CaCO₃ in their cement (e.g. van Dijk et al., 2017). These cultured genera allow to compare three different foraminiferal groups related to different shell structure (perforate, imperforate and agglutinated) and test building mechanism. *Quinqueloculina* and *Textularia* are shallow-water genera commonly distributed along the anthropic coastal areas (Murray, 2006).

Monospecific cultures were maintained in plastic Falcon tubes (volume 50 mL) with seawater filtered with a 0.42 mm membrane filter at pH of about 8.0, adjusted in salinity of 35 at room temperature (approximately 50 mL per vial), to obtain reproduction and subsequent growth of the juveniles populations. In order to reduce the fast increase of salinity and the accumulation of organic waste products and other organisms (i.e. bacterial biofilm), we refilled the culturing tubes by replacing about 20–40% of original seawater. Measures of salinity, temperature, and pH were performed weekly and correction of salinity was performed adding deionized water when necessary. All cultures were fed every week with a few drops of freeze-killed concentrated algae (*Isochrysis galbana*, Haptophyta widely cultured for use in the aquaculture industry, e.g. Camacho-Rodriguez et al., 2020; Kaparapu et al., 2018; Shields et al., 2012).

For the experiment, we selected individuals in a size range from 90 to 150 µm in order to have adult specimens and not juvenile forms which could bias the taxonomic identification. Then, we observed them under the stereomicroscope NIKON SMZ-U to check their vitality. In particular, vitality was assessed by the presence of sediment particles close to the aperture and/or yellow/brown colour; these samples were further observed under the optical microscopy Nikon Eclipse E 600 POL with phase contrast, in order to further detect pseudopodial activity (Fig. 2).

2.2. Experimental design

2.2.1. Preparation of the stock solution

Toxicity test methods followed US Environmental Protection Agency (EPA) acute protocols (USEPA, 2009). The stock solution for the toxicity test was made by adding 32 naturally smoked cigarette butts (smoked filter + tobacco) in 1 L of artificial seawater (deionized water + Instant ocean®) for 48 h. Soon after, the stock solution was sieved using 32 µm mesh in order to remove the cigarette butts remains. A “naturally smoked” cigarette is defined as a cigarette that was actually smoked by people, extinguished in cigarette disposal units and collected within 1 week from the deposition. All cigarettes contained intact filters, covering paper, and a small amount of unsmoked and burned tobacco. Artificial seawater was prepared adding 1 L deionized water and 375 g of Instant Ocean® and after overnight stabilization, yield a pH range between 8.0 and 8.4 and a salinity range of 35–37 ppt. Prior to the selection of individuals for the experiment, following the APAT-IRSA protocol (APAT-IRSA, 2003) a preliminary test, with 5 concentrations (obtained by two-fold serial dilution of the stock solution) was performed, in order to obtain the best concentrations to work with. The test was carried out by using 5 individuals for each concentration (1 replicate) plus the control. After 48 h of exposure, the concentrations for the

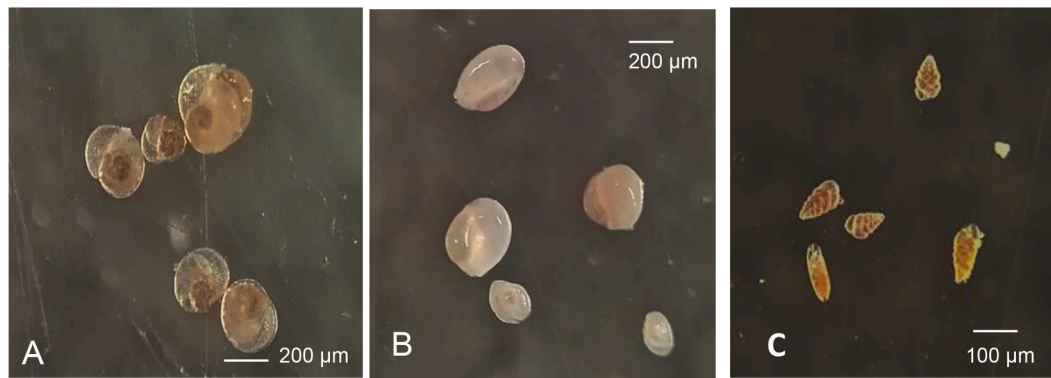


Fig. 1. Foraminiferal species from laboratory cultures. A) *Rosalina globularis*, B) *Quinqueloculina* spp. and C) *Textularia agglutinans*.

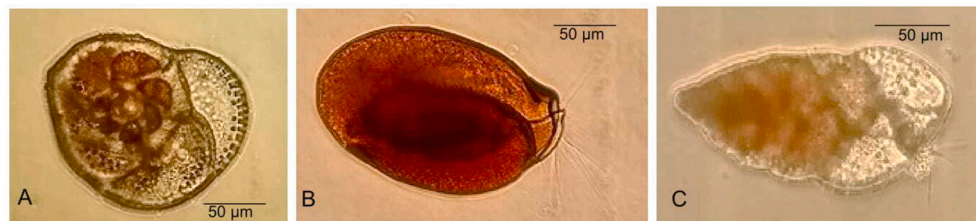


Fig. 2. Alive foraminifera, images from optical microscopy Nikon Eclipse E 600 POL with phase contrast. A) yellow/brown cytoplasm of *Rosalina globularis*, B) pseudopodial activity of *Quinqueloculina* spp., and C) pseudopodial activity of *Textularia agglutinans*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

final test were selected, considering that the lowest concentration should cause at most 5% death while your highest concentration should cause 95–100% death.

2.2.2. Foraminiferal LC_{50} test

The foraminiferal specimens belonging to the 3 genera, previously living-checked and measured, were picked from monospecific cultures with a fine and moistened brush and inserted in the tubes previously prepared with the dilution series. For all 3 genera considered, 4 replicates for the 5 used concentrations were taken. Each replicate contained 5 individuals, for a total of 20 organisms per concentration, plus one control concentration (nominally 0 cigarette butt/L) (Fig. 3, Table 1) according to Slaughter et al. (2011) and APAT-IRSA (2003) protocol. The test (LC_{50} - 48 h) was run under laboratory controls: tubes on the laboratory bench, water quality readings (temperature, salinity, and pH) were performed before and after the experiment and a light-dark (12

h–12 h) cycle by artificial light was maintained, while survival count only after 48 h.

After the experimental treatments, all foraminiferal specimens were removed from experimental tubes by brush and, following the Ross and Hallock method (2018), they were rinsed with clean seawater three times to remove residual of cigarette butts solution. Then, they were put on the laboratory bench in glass Petri dishes with 15 mL filtered seawater and 0.2 µL (0.3 µM) of fluorescent probe Cell Tracker TM Green CMFDA (CTG) at 37 PSU salinity for 30 min. Soon after, the foraminifera were again rinsed with clean seawater to remove any remaining CTG and checked for cellular activity using a fluorescence binocular microscope. The foraminifera were then allowed to recover for 72 h in clean seawater, following the protocols developed by Ross and Hallock (2012). After 72 h, colour and activity of each individual were visually assessed to identify possible quiescent organisms. In fact, a fundamental point in these kinds of studies is to distinguish dead foraminifera versus

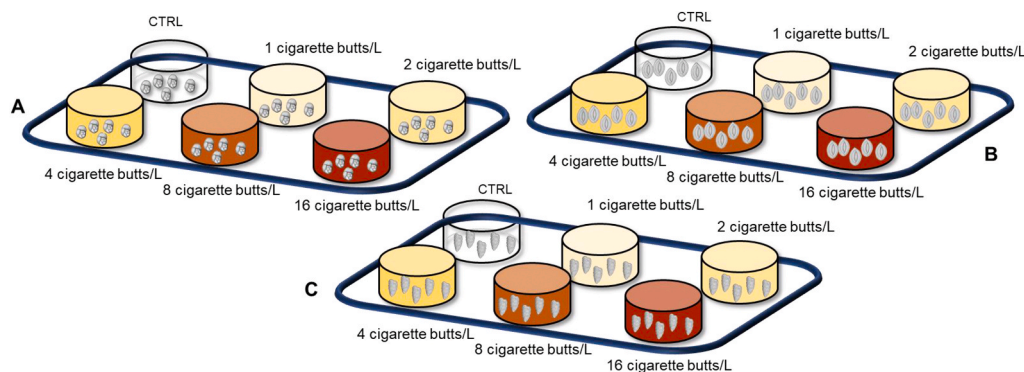


Fig. 3. Schematic design of the experiment. The plates represent the three different species: A) *Rosalina globularis*; B) *Quinqueloculina* spp; C) *Textularia agglutinans*. The petri dishes denote the cigarette butts concentration (16, 8, 4, 2 and 1 cigarette butts/L), whereas colours refer to real colour observed when the dilution series was performed. In the figure are not represented all the replicates considered for the experiment (see section 2.2.2.), but it is only an example related to 1 replicate and control. The cigarette butts concentrations, reported in the figure, correspond respectively to 31.25, 15.63, 7.82, 3.91, 1.96 mg of nicotine/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Foraminiferal mortality after LC₅₀-48 h and 72 h recovery. To discriminate death from those quiescent, individuals were labelled with the fluorescent probe Cell Tracker TM Green CMFDA (CTG). For concentrations see caption Fig. 3.

Cigarette butts/L		<i>R. globularis</i>		<i>Quinqueloculina</i> spp.		<i>T. agglutinans</i>	
		48 h	72 h	48 h	72 h	48 h	72 h
16	R1	5	5	5	5	2	4
	R2	5	5	5	5	5	5
	R3	5	5	3	5	2	5
	R4	5	5	5	5	n.d.	n.d.
8	R1	4	4	4	5	3	4
	R2	4	4	5	5	1	2
	R3	5	5	4	4	2	4
	R4	5	5	5	5	n.d.	n.d.
4	R1	3	5	2	5	3	4
	R2	3	5	3	3	5	5
	R3	3	4	3	5	3	5
	R4	5	5	2	3	n.d.	n.d.
2	R1	4	4	3	4	4	4
	R2	5	5	2	5	2	4
	R3	5	5	2	3	5	5
	R4	5	5	3	4	n.d.	n.d.
1	R1	5	5	2	5	1	2
	R2	5	5	3	4	4	4
	R3	5	5	5	5	2	5
	R4	5	5	4	5	n.d.	n.d.
CTRL	R1	0	0	0	0	0	0
	R2	0	0	0	0	0	0
	R3	0	0	0	0	0	0
	R4	0	0	0	0	n.d.	n.d.

survivors. Previous work (Ross and Hallock, 2016; Ross, 2012) reported that foraminifera can survive a stressful condition by entering a low activity dormant state, looking to be dead. This life-strategy could affect the experiment results causing underestimated survivors, therefore the CTG was used to overcome this problem. CTG is a non-terminal, non-fluorescent-probe that can be cleaved by non-specific esterase common to living cells, producing a fluorescent compound, fluorescein, visible using a fluorescent microscope (Bernhard et al., 2006). The requirement of esterase activity means that a cell must be alive to produce fluorescence. In this regard, recent studies (i.e. Pucci et al., 2009; McIntyre-Wressnig et al., 2014; Ross and Hallock, 2018) demonstrated that CTG is a good method for determining mortality in bioassay and other laboratory experiments. For these reasons, after the experiment, all foraminiferal species CTG labelled were the endpoint value and data were analysed to identify the median lethal effect concentration (LC₅₀), the concentration of cigarette butt leachate resulting in 50% mortality, and concentration-response curve. The calculation of LC₅₀ values was done by an online system through the four parameters logistic equation, which corresponds to the dose-response curve with the slope of the variable slope (Quest Graph™ LC₅₀ Calculator, <https://www.aatbio.com/tools/lc50-calculator>). The entire protocol related to the foraminiferal LC₅₀ test is available as supplementary material (S1).

2.3. Chemical analysis of stock solution by gas chromatography–mass spectrometry (GC-MS) analysis

In order to detect and quantify the nicotine present in the experimental treatments, 20 mL of the same 32 cigarette butts/L stock solution employed in the experimental treatments and prepared as previously described have been lyophilized. The dried residue has been reconstituted with 25 mL of MeOH before the GC-MS analysis. Gas chromatography analyses were performed on Agilent equipment (GC 6890 N) with single quadrupole mass spectrometer detector (MSD 5975) and split/splitless injector. Both systems were controlled by MSD ChemStation (Agilent Technologies, Inc.). An Agilent capillary column, type HP-5ms (5%-phenyl-methylpolysiloxane (30 m, 0.25 mm i. d., 0.10 µm film thickness) was used. The oven temperature program was started at 45 °C held for 2 min, increased to 300 °C at a rate of 15 °C/min, held for

2 min. Total analysis time was 21 min. The split/splitless injector was used with injector temperature 300 °C and split ratios 30:1. Helium was used as a carrier gas, with a flow rate of 1.0 mL/min. Inject volume was 1 µL, solvent delay 2.0 min, and MSD ionization voltage 70 eV. Data was collected in SCAN (29–450 m/z). The nicotine concentration was calculated by using an external calibration curve.

2.4. Fourier Transform infrared analyses

To better quantify the relative loss in calcium carbonate that the 3 foraminiferal genera underwent, Fourier Transform infrared (FTIR) imaging was used. Foraminiferal samples were measured at SISSI beamline, biochemical and life sciences branch (SISSI-Bio) at Elettra Sincrotrone Trieste. The sample's pool contained 7 individuals of *R. globularis*, 7 individuals of *Quinqueloculina* spp. and 7 individuals of *T. agglutinans*. Among them 1 individual for each genus was taken from the control (CTRL- 0 cigarette butt/L). 3 replicates for each genus were taken from the sublethal concentration that corresponded with 1 cigarette butt/L for *R. globularis* and 2 cigarette butts/L for both *Quinqueloculina* spp. and *T. agglutinans*. At last, 3 replicates for each genus were taken from the LC₅₀ concentration (see section 3.1) (Table 2). Samples were inspected under an optical microscope, Zeiss Stemi 305, and then transferred onto the Diamond compression cell (ST Japan). There, the two halves of the cell were placed one on top of the other and the samples slowly compressed between the two diamonds. When the sample is pulverized and compressed enough to be measured in transmission mode, the cell was opened and put under the Hyperion 3000 optical/infrared microscope (Bruker Optics) to be measured. For each sample, the whole imprint left on the face of the compression cell was measured in imaging mode with a 64 × 64 pixel Focal Plane Array (FPA), collecting 128 scans at 4 cm⁻¹ spectral resolution. The pixel size acquired with 15× IR/VIS objective/condenser Cassegrain optics is 2.66 × 2.66 µm.

After all the data were acquired, they were corrected in OPUS software (Bruker Optics) for water vapor and CO₂ contributions to the spectra. In order to evaluate the effect of the cigarette leakage to the foraminifera, band integrals were calculated: 1713–1610 cm⁻¹ for proteins, representative of cytoplasm, 3000–2800 cm⁻¹ for lipids, 2660–2435 cm⁻¹ overtone of the calcium carbonate, and 1195–985 cm⁻¹ for phosphates, silicates, and carbohydrates. Results of the band's integration processes were tabled in Excel (Microsoft Corp.), the negative values were removed, and then averaged for every sample in order to allow for comparison. They were then plotted in Origin (Origin labs).

3. Results

3.1. Chemicals in the stock solution

The presence of nicotine in the cigarette butt infusions has been

Table 2

Foraminiferal samples measured at SISSI beamline. The sample's pool contains in total 7 individuals of each genus (*Rosalina globularis*, *Quinqueloculina* spp., *Textularia agglutinans*), so 1 individual for each replicate was considered. The numbers in parenthesis refer to the concentration of cigarette butts/L. For concentrations see caption Fig. 3.

	<i>R. globularis</i> (Cigarette butts/L)	<i>Quinqueloculina</i> spp. (Cigarette butts/L)	<i>T. agglutinans</i> (Cigarette butts/L)
Control (CTRL)	R1 (0)	R1 (0)	R1 (0)
Sublethal concentration	R1 (1)	R1 (2)	R1 (2)
	R2 (1)	R2 (2)	R2 (2)
	R3 (1)	R3 (2)	R3 (2)
Lethal concentration (LC ₅₀)	R1 (1.9)	R1 (7.2)	R1 (5.6)
	R2 (1.9)	R2 (7.2)	R2 (5.6)
	R3 (1.9)	R3 (7.2)	R3 (5.6)

determined by GC-MS analysis on a known volume of the stock solution (32 cigarette butt/L), employed in the preparation of the experimental treatments, lyophilized and re-dissolved in methanol as described in Materials and Methods section. Because the five exposure levels (16, 8, 4, 2, and 1 cigarette butt/L) have been obtained by serial dilution of the filtered stock solution, we performed the nicotine determination only on the concentrated stock solution. The GC-MS revealed the prevailing presence of (S)-3-(1-Methylpyrrolidin-2-yl)pyridine ((S)-nicotine) as evidenced by both the GC retention time (Fig. 7) and the presence of the proper molecular ion $[M-H]^+$ (m/z 161) with the relative fragmentation into the product ions (m/z 133, 84) (Fig. 8). The assignment of the principal peak to nicotine has been made by matching its retention time and mass spectral data with retention time and fragmentation patterns of a nicotine reference standard. By using an external calibration curve, a nicotine concentration of 62.5 mg/L was determined in the stock cigarette butt infusion. The concentration of the solutions employed in the following experiments obtained by two-fold serial dilution of the stock solution were 16 (31.25 mg of nicotine/L), 8 (15.63 mg of nicotine/L), 4 (7.82 mg of nicotine/L), 2 (3.91 mg of nicotine/L), 1 (1.96 mg of nicotine/L) cigarette butts/L.

3.2. Foraminiferal LC_{50} values

The seawater quality parameters (T, S, and pH) measured during the experiment, are reported in (Table 3). Temperature and salinity values remain unvaried during the experiment, while the pH displays a progressive decrease with increasing cigarette butts concentration. The results of LC_{50} test of cigarette butt leachates for foraminifera, after a 30-min CTG treatment and a 72 h incubation, to discriminate death individuals from those quiescent, are shown in Table 1. Leachate from smoked cigarette butts (SCB) was found to be toxic for all the foraminiferal genera investigated. *Rosalina globularis* was found to be the most sensitive species with a LC_{50} of 1.9 cigarette butts/L, followed by *T. agglutinans* with 5.6 cigarette butts/L, and *Quinqueloculina* spp. with 7.2 cigarette butts/L (Fig. 4). The concentration-response curve shows that both the calcareous perforate *R. globularis* and the imperforate genus *Quinqueloculina* spp. respond in a different way compared to the agglutinated species, *T. agglutinans* (Fig. 5). The viability of both calcareous taxa declines as the cigarette butts concentration increases (4 cigarette butts/L) without recovery, while the viability of the

agglutinated species decreases at medium concentrations (4 cigarette butts/L) and then recovers at high leachate concentrations (8 and 16 cigarette butts/L). Instead, all individuals from laboratory controls survived, as required by EPA protocol for test validity (90% or greater, USEPA, 2009) and no-abnormalities were observed in the controls (Table 1).

Qualitative analysis shows that, at the higher concentrations (4, 8, 16 cigarette butts/L) and for every replicate, all foraminiferal taxa are exposed to partial shell dissolution regardless of the LC_{50} values. Some examples are reported in Fig. 6. In *R. globularis* only the last chamber is systematically prone to dissolution (Fig. 6A). *Quinqueloculina* spp. individuals show a complete dissolution of the imperforate test and the disorganised cytoplasm is clearly visible with the presence of dark inclusions (Fig. 6B). For *T. agglutinans* the complete shell dissolution is partially observable only at the last chambers where also the cytoplasm is disorganised showing the presence of stercomata and dark inclusions (Fig. 6C).

3.3. FTIR

Fourier Transform infrared (FTIR) data are shown in Fig. 9 as histograms of selected integral band ratios for each of the 3 genera. Band integrals on proteins and lipids can give information of the status of the organic content of the foraminifera, in terms of cytoplasm average composition. The band of calcium carbonate and the one of phosphates/carbohydrates (comprehending also a signal from chitin), can provide more info about the shell composition. The variations of the key macromolecules composing the foraminifera are here presented normalized to the protein content, in order to remove any bias due to the difference of thickness within the population of the inspected samples.

Fig. 9A and B shows the values for *R. globularis* and *Quinqueloculina* spp. These two genera are more affected by the pollution of naturally smoked cigarettes with respect to *T. agglutinans*. In the case of *T. agglutinans* (Fig. 9C), it is possible to observe that the overall metabolic profile is not heavily altered by the presence of cigarette butts in sea-water: lipids over protein exhibit a steady trend, a hint of a minor effect on the cell organic components. Considering the shell composition, instead, we see an increase in both carbohydrates and calcium carbonate signals. Between the two calcareous taxa, the most affected is *Rosalina globularis*, which shows decalcification of the shell already at

Table 3

Seawater quality parameters (temperature, salinity and pH) measured at the beginning and at the end of the experiment. In the table are reported the values for each genus, concentration and replicate. For concentrations see caption Fig. 3.

Cigarette butts/L		<i>R. globularis</i>						<i>Quinqueloculina</i> spp.						<i>T. agglutinans</i>					
		Temperature (°C)		Salinity (PSU)		pH		Temperature (°C)		Salinity (PSU)		pH		Temperature (°C)		Salinity (PSU)		pH	
		0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h
		0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h
16	R1	24.5	24.5	37.5	37.5	7.20	6.90	24.5	24.5	37.5	37.5	7.18	6.90	24.5	24.5	37.5	37.5	7.22	6.90
	R2	24.5	24.5	37.5	37.5	7.21	6.82	24.5	24.5	37.5	37.5	7.16	6.82	24.5	24.5	37.5	37.5	7.14	6.82
	R3	24.5	24.5	37.5	37.5	7.22	6.66	24.5	24.5	37.5	37.5	7.18	6.66	24.5	24.5	37.5	37.5	7.16	6.66
	R4	24.5	24.5	37.5	37.5	7.26	6.74	24.5	24.5	37.5	37.5	7.14	6.74	24.5	24.5	37.5	37.5	n.d.	n.d.
8	R1	24.5	24.5	37.5	37.5	7.86	7.24	24.5	24.5	37.5	37.5	7.85	7.24	24.5	24.5	37.5	37.5	7.83	7.05
	R2	24.5	24.5	37.5	37.5	7.93	7.33	24.5	24.5	37.5	37.5	7.86	7.33	24.5	24.5	37.5	37.5	7.81	7.12
	R3	24.5	24.5	37.5	37.5	7.94	7.30	24.5	24.5	37.5	37.5	7.81	7.30	24.5	24.5	37.5	37.5	7.86	7.10
	R4	24.5	24.5	37.5	37.5	7.93	7.28	24.5	24.5	37.5	37.5	7.89	7.28	24.5	24.5	37.5	37.5	n.d.	n.d.
4	R1	24.5	24.5	37.5	37.5	8.19	7.68	24.5	24.5	37.5	37.5	8.22	7.57	24.5	24.5	37.5	37.5	8.18	7.58
	R2	24.5	24.5	37.5	37.5	8.22	7.55	24.5	24.5	37.5	37.5	8.23	7.70	24.5	24.5	37.5	37.5	8.18	7.63
	R3	24.5	24.5	37.5	37.5	8.16	7.52	24.5	24.5	37.5	37.5	8.22	7.63	24.5	24.5	37.5	37.5	8.21	7.63
	R4	24.5	24.5	37.5	37.5	8.18	7.42	24.5	24.5	37.5	37.5	8.13	7.49	24.5	24.5	37.5	37.5	n.d.	n.d.
2	R1	24.5	24.5	37.5	37.5	8.30	8.06	24.5	24.5	37.5	37.5	8.33	8.03	24.5	24.5	37.5	37.5	8.33	8.09
	R2	24.5	24.5	37.5	37.5	8.34	8.16	24.5	24.5	37.5	37.5	8.32	7.93	24.5	24.5	37.5	37.5	8.36	8.21
	R3	24.5	24.5	37.5	37.5	8.33	8.13	24.5	24.5	37.5	37.5	8.32	8.00	24.5	24.5	37.5	37.5	8.35	8.08
	R4	24.5	24.5	37.5	37.5	8.35	8.08	24.5	24.5	37.5	37.5	8.32	8.05	24.5	24.5	37.5	37.5	n.d.	n.d.
1	R1	24.5	24.5	37.5	37.5	8.36	8.30	24.5	24.5	37.5	37.5	8.38	8.32	24.5	24.5	37.5	37.5	8.33	8.35
	R2	24.5	24.5	37.5	37.5	8.37	8.31	24.5	24.5	37.5	37.5	8.38	8.30	24.5	24.5	37.5	37.5	8.37	8.29
	R3	24.5	24.5	37.5	37.5	8.39	8.36	24.5	24.5	37.5	37.5	8.39	8.31	24.5	24.5	37.5	37.5	8.38	8.03
	R4	24.5	24.5	37.5	37.5	8.37	8.16	24.5	24.5	37.5	37.5	8.38	8.22	24.5	24.5	37.5	37.5	n.d.	n.d.

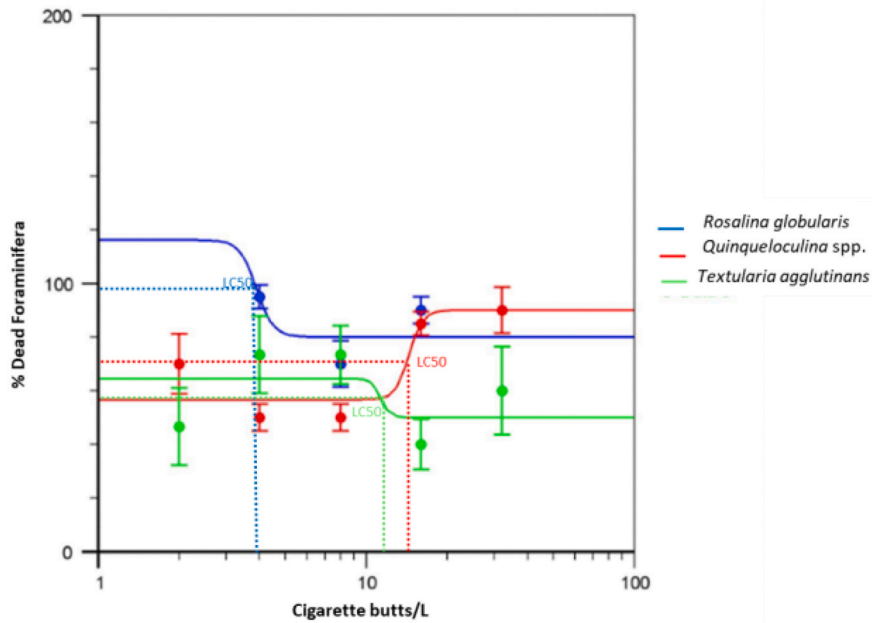


Fig. 4. LC₅₀ test result for the three foraminiferal species: *Rosalina globularis* (blue), *Quinqueloculina* spp. (red), *Textularia agglutinans* (green). For concentrations see caption Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

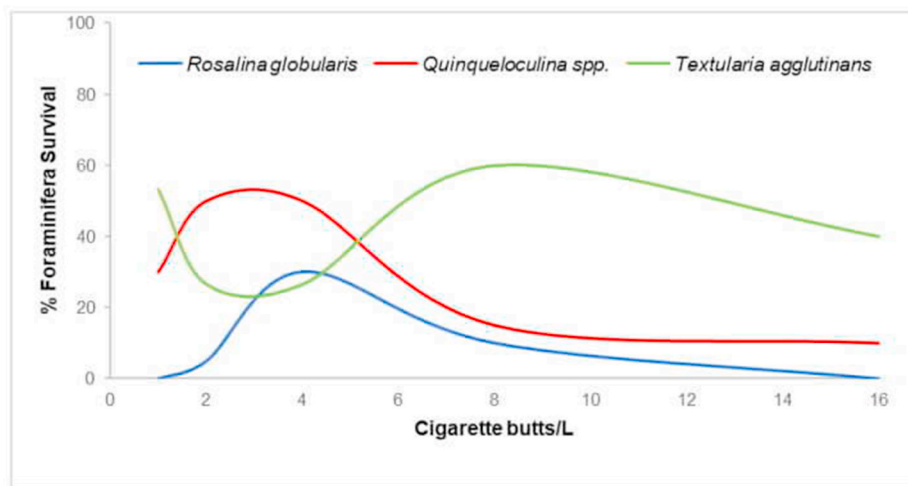


Fig. 5. The cigarette butts leachate concentration vs percentage of survived foraminifera for the three studied species: *Rosalina globularis* (blue), *Quinqueloculina* spp. (red) and *Textularia agglutinans* (green). For concentrations see caption Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

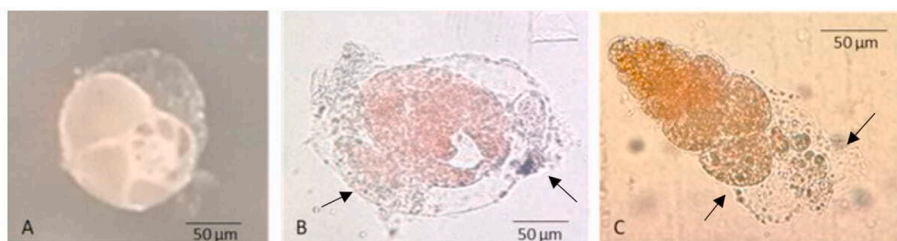


Fig. 6. Examples of shell dissolution in: A) *Rosalina globularis*, B) *Quinqueloculina* spp.; is clearly visible the decalcification of the imperforate test and the alteration of cytoplasm with the presence of dark inclusions indicated by the arrows and C) *Textularia agglutinans*. The complete shell dissolution is partially observable only at the last chambers where also the cytoplasm is disorganised showing the presence of stercomata and dark inclusions, indicated by the arrows..

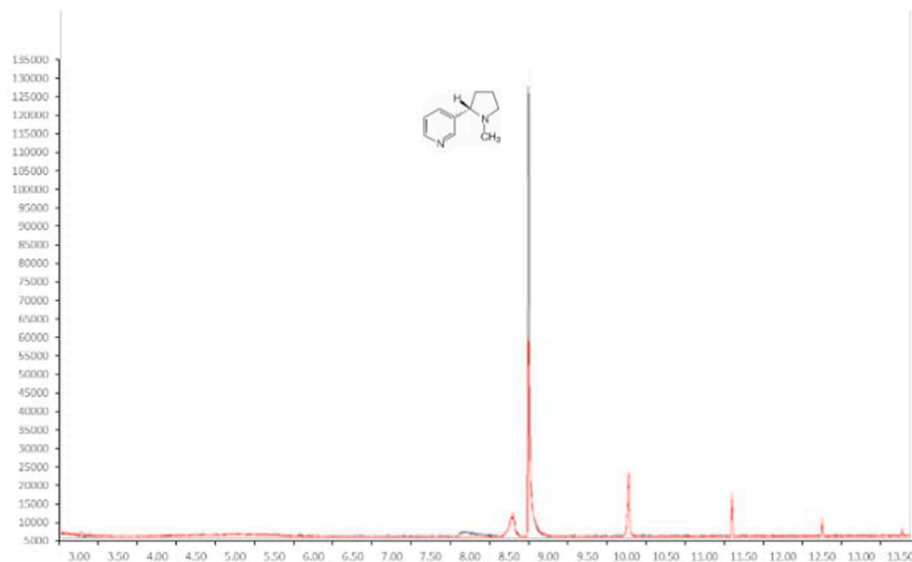


Fig. 7. Superimposed GC/MS chromatogram of cigarette butt infusion sample (red) and nicotine standard (black).

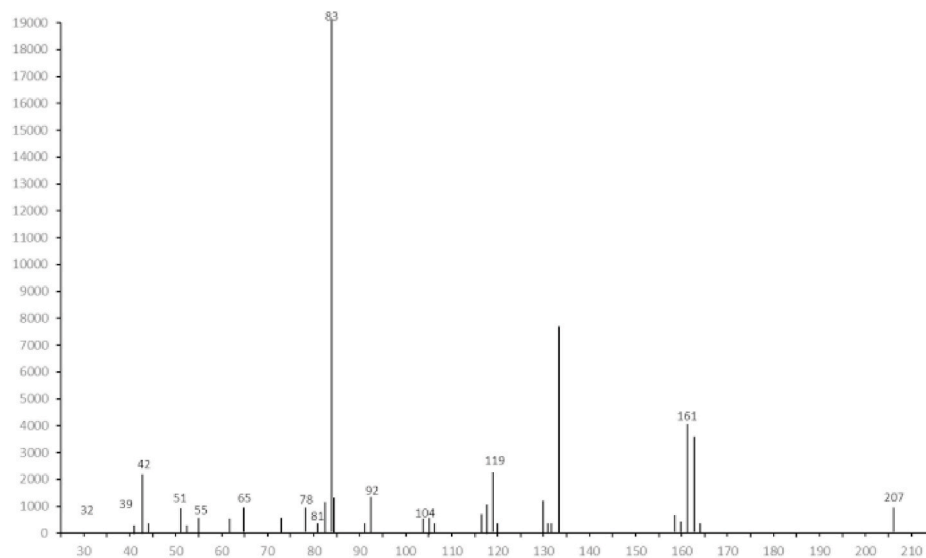


Fig. 8. Mass spectrum of peak with retention time 8.820 min, matching fragmentation patterns of the nicotine standard.

low concentration of cigarette butt leachate and alteration of the overall metabolic profile with a strong effect on the cell organic components. The same is observed for *Quinqueloculina* spp., even if the magnitude of the variation is lower (although not statistically significant due to the high intra-sample variability). About the carbohydrates over proteins' bands ratio, indicative of the variations of chitin within the shell composition, *Quinqueloculina* spp. follows the trend of *T. agglutinans*, increasing the signals of this chemical species as the concentration of cigarette butt leachate increases in the solution; in *R. globularis*, instead, these signals decrease as the cigarette butts pollution of the medium increases.

4. Discussion

So far, no studies considered the impact of smoked cigarette butts on benthic foraminifera, and in general, also for other organisms (from the simplest, like vibrio to the most evolved, like fishes), the literature is not that wide. Previous studies have shown that leachate cigarette butts can be toxic to other aquatic organisms. For example, a study conducted by

Register (2000) found that leachate concentrations between 0.125 and 0.25 cigarette butts/L after a 48-h experiment of LC₅₀, from smoked cigarette butts with tobacco (no filter), were acutely toxic for *Daphnia magna*. The experiment was also conducted with leachate from smoked cigarette filters (no tobacco) that was found to be toxic between 1 and 2 cigarette butts/L (48 h LC₅₀). These experiments demonstrated how the remaining tobacco in the cigarette butts together with filters contributed to their toxicity. According to Register, 2000, Slaughter et al., 2011, in an experiment with marine fishes (*Atherinops affinis* and *Pimephales promelas*), it is demonstrated a progressive increase in toxicity from artificially Smoked Filters (no tobacco) (SF) to artificially Smoked Cigarette Butts (smoked filter + tobacco). The SCB produced an LC₅₀ of 1 cigarette butt/l for both marine fishes while the toxic concentrations of SF were 1.8 cigarette butts/l for *A. affinis* and 4.3 cigarette butts/L for *P. promelas*. However, other authors investigated the effective concentration (EC₅₀). For example, Warne et al. (2002) found leachates from smoked cigarette butts, smoked cigarette filters, and unsmoked cigarette filters to be acutely toxic for *Vibrio fischeri* at 0.58 cigarette butts/L (30-min EC₅₀ (bioluminescence)) and Micevska et al. (2006) supported

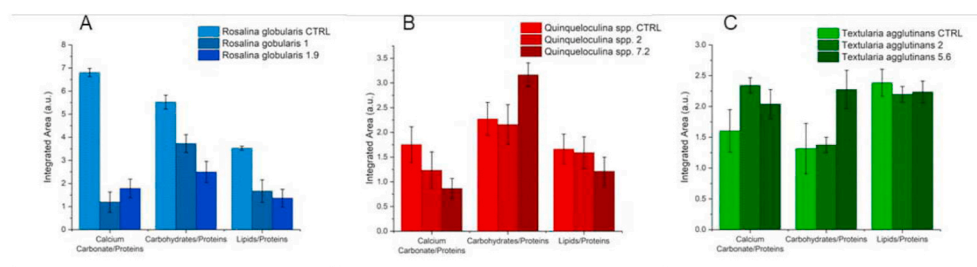


Figure 9. Histograms of the ratios Calcium carbonate/Proteins, Carbohydrates/Proteins and Lipids/Proteins for the three foraminiferal species considered. From left to right in order of sensitivity to the presence of cigarette leachates in the growth medium. A. In shades of blue, the bar chart represents the ratios of the band's integral average values for the *Rosalina globularis*. B. In shades of red, same representation for *Quinqueloculina* spp. data. C. In shades of green, bar chart representing the ratios of the band's integral average values for the *Textularia agglutinans*, the black bar represents standard error. For all the cigarette filters increase in concentration is represented by the darkening of the colour of the bar. Legend numbers are related to control, sublethal and lethal concentration of cigarette butts/L for the three species as specified in the section 2.4 and in the Tab. 2. For concentrations see caption Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

this finding with an artificially smoked cigarette butts leachate EC₅₀ (30-min bioluminescence) between 0.3 and 2.7 cigarette butts/L.

Our results show that cigarettes leachate was acutely toxic to all considered foraminiferal genera at different concentrations for each species (Fig. 4). In fact, the population of *R. globularis* dies at a concentration of 1.9 cigarette butts/L, *Quinqueloculina* spp. at 7.2 cigarette butts/L and *T. agglutinans* at 5.6 cigarette butts/L. These high values, compared to those discussed above, may be related to the fact that the present study considers only human-smoked cigarettes containing both filters and residual tobacco, as they are found in the environment. Littered cigarette butts found in the environment encompass a wide range of brands with varying amounts of tobacco and, therefore, toxic substances left on the filters. LC₅₀ evaluations by other authors were determined using different methodology: for example, Register (2000), used discarded cigarette butts and conducted each experiment with the single components of cigarette butts (remaining tobacco or filters); Slaughter et al. (2011), in experiments on marine fishes, used artificially smoked cigarettes (with and without tobacco) and unsmoked cigarette filters; Micevska et al. (2006) investigating the variation in the acute toxicity of artificially smoked cigarette butts to *Ceriodaphnia* cf. *dubia* and *Vibrio fischeri*, considering for the experiments different brands and separating the result relatively to each one. All these methods probably produce a leachate potentially less toxic than the one we produced resulting from a mixture of different brands and therefore different concentrations of nicotine and other toxic chemicals. Despite that, it is evident that foraminifera appear to be more resistant than other species belonging to other phyla with different taxa-specific responses (Table 4).

In particular, foraminiferal reaction to cigarette butt leachate could be due to the co-presence of different compounds. Previous studies report the qualitative and quantitative composition of cigarette butts

and the most potentially hazardous constituents of smoke are tar, nicotine, carbon monoxide, nitric oxide, hydrogen cyanide, acrolein, benzo(a)pyrene, and N-nitrosamines, however nicotine is by far the most abundant. Wright et al. (2015) reported that the nicotine concentration in the smoked cigarette filters leachate (8 filters/L), after 96 h exposure, was 694 ng/mL. In our preparation, the nicotine content is 62.5 mg/L. That is approximately two orders of magnitude greater than that reported by Wright et al. (2015). In particular, the GC-MS analysis shows the prevailing presence of S-nicotine (Figs. 7 and 8). This nicotine enantiomer represents the most abundant and active compound in nature, which binds to neuronal nicotinic acetylcholine receptors (NACHRs) each of which has specific pharmacology and physiology (Gotti et al., 1997) affecting the health of the cell. This suggests that this prevalent concentration of S-nicotine is the main factor responsible for the high foraminiferal LC₅₀ values.

However, the LC₅₀ values obtained in the experiment using foraminifera not only are high compared to other organisms (i.e. *Vibrio fischeri*, *Daphnia magna* and fishes) but also varies among the foraminiferal taxa. In our study, the foraminiferal survival results (Fig. 5) show how the vitality curve of both calcareous foraminifera *R. globularis* and *Quinqueloculina* spp., perforate and imperforate respectively, highlight the same trend; starting from 4 cigarette butts/L a decalcification shell effect is recorded (Fig. 5) that does not correspond to individuals death. Instead, starting from 8 cigarette butts/L the death of individuals for both calcareous genera is observed and in the case of *R. globularis*, at the highest concentration (16 cigarette butts/L), the curve reaches zero. The agglutinated species shows a different trend compared to the calcareous ones. In fact, at the highest cigarette butt concentrations (16 cigarette butts/L) *T. agglutinans* shows partially dissolved test of the last chambers probably due to the consequent dissolution of the calcitic cement, while cytoplasm seems still well organized and it fills the rest of the shell (Figs. 6C and 9C). This behaviour evidences that the effect of cigarette butts is both in relation to toxic effects on the entire organism and decalcification. Furthermore, test dissolution has never been observed in other organisms because the other phyla discussed above do not biocalcify.

The biomineralization process in foraminifera is a complex mechanism not completely resolved yet. Last biomineralization model in perforate foraminifera (Tyszk et al., 2019) suggests a semi intracellular biomineralization pattern known from other unicellular calcifying and silicifying organisms. This implies, for sure, a regulatory function in the calcification process driven by the cell. For this reason, we suggest that

Table 4

LC50 values for leachates from smoked cigarette butts for foraminifera obtained in this study. LC50 values for other organisms are given for comparison. For concentrations see caption Fig. 3.

Species (LC ₅₀)	Cigarette butts/L	References
<i>Rosalina globularis</i>	1.9	Current study
<i>Quinqueloculina</i> spp.	7.2	Current study
<i>Textularia agglutinans</i>	5.6	Current study
Topsmelt	1.1	Slaughter et al. (2011)
Fathead minnow	0.97	Slaughter et al. (2011)
Daphnid (<i>D. magna</i>)	0.125–0.25	Register et al. (2000)

the different response might be due to a different biomineralization process and test building mechanism (i.e. the arrangement of calcite crystal in the tests, high vs low Mg content, selection of foreign particles) and species biology and ecology (i.e. whether the species hosts symbionts). In particular, the perforate and imperforate species adopt a similar physiological strategy to precipitate calcite even though they have a different calcification pathway (de Nooijer et al., 2009). In this regard, culture experiments on calcification of the perforate foraminifera *Cibicides lobatulus* showed that it increases the pH in the carbonate pool during chamber formation with the possible diffusion of metabolic CO₂. The model for the imperforate foraminifera considered that specimens elevate the pH during calcite precipitation and subsequently the same pH decreases during the chamber formation (de Nooijer et al., 2009). Instead, the agglutinated foraminifera secrete an organic matrix holding together a suite of particles, silicates or carbonates, of various sizes and shapes.

In this context, the FTIR data supported our morphological evidence of decalcification and analysis evidenced that the 3 genera show three different behaviours in response to the presence of cigarette butts. At the extremes, we have *T. agglutinans*, almost not affected by the presence of the pollutant, and *R. globularis*, whose biochemical composition of the cell is altered already at low concentration of cigarettes together with carbonatic shell dissolution (Fig. 9C). *Quinqueloculina* spp. presents an intermediate response, still showing clear decalcification of the shell and decrease in the overall cell organic components, but also trying to respond to the induced stress pollution increasing the chitin/carbohydrate composition of the shell to prevent its complete dissolution. The first hypothesis to formulate refers to the co-presence of dissolved substances with acidic properties, in fact, the pH in the solution decreased up to 6.81 after 48 h. Toyofuku et al., 2017, demonstrated how a relatively moderate decrease in pH (6.9) may not affect foraminiferal calcification because they are thought to promote calcification by elevating their intracellular pH (Toyofuku et al., 2016; de Nooijer et al., 2009) and this considerably varies among species as they adapt to different habitats (van Dijk et al., 2017; Fujita et al., 2011). As a general rule, Miliolids (imperforate foraminifera) as *Quinqueloculina* possess high magnesium calcite tests (Bentov and Erez, 2006) which are more vulnerable to the effect of ocean acidification than calcareous perforate *Rosalina* (Uthicke et al., 2013; Fujita et al., 2011; Kuroyanagi et al., 2009). In addition, the genus *Rosalina* has monolamellar and low magnesium calcite tests (Bentov and Erez, 2006). The agglutinated species build a shell by affixing particles within a matrix of organic compounds or calcium carbonate (Bender and Hemleben, 1988). As for agglutinated foraminifera, previous studies examining the foraminiferal assemblages in relation to ocean acidification from natural or experimental conditions reported that they are more resilient than calcareous species (Pettit et al., 2013; van Dijk et al., 2017). In particular, van Dijk et al. (2017) conducted an experiment on four different species of agglutinated foraminifera (among them the genus *Textularia* and the species *Textularia tenuissima*) incubated in an artificial sediment of pure glass spheres, at high pCO₂ concentration (2000 ppm, that means pH around 7.2 and therefore definitely higher than the values we obtained) to evaluate the combined impacts of ocean acidification and dysoxia on agglutinated foraminifera. They demonstrated that under these conditions none of the investigated *T. tenuissima* produced cement composed of calcium carbonate, without potential effects from calcification stress in their physiological response, instead, evidencing unexpected potential implications for the classification in the carbonate-cement producing Order Textularia (Loeblich and Tappan, 2015).

Our FTIR results are in partial agreement with van Dijk et al. (2017) as *T. agglutinans* seems to promote the production of carbonate-cement to avoid the breaking up of the test. Only the last chambers undergo this process, but the species increases also the production of chitin within the shell composition to strengthen the test resistance to cigarette butts pollution. We suggest that the increase of the signals of chitin in the test might represent the survival strategy behaviour for this taxon

that lives also in stressed conditions of acidification and dysoxia induced in laboratory experiments (van Dijk et al., 2017). Also, the cell of *T. agglutinans* is less altered by cigarette butt pollution and because of this behaviour the species is clearly, the most resistant among the three analysed.

Therefore, based on our results, we suggest that a significant pH reduction, like that recorded in this study, may be responsible for a reduction of the ability of organisms to increase the amount of energy required to elevate intracellular pH to levels required for calcification in both calcareous species investigated. This may cause alterations in the foraminiferal physiology, leading to the cellular death in both calcareous species (*R. globularis* and *Quinqueloculina* spp.) at the highest concentration of leachate. Instead, the vitality and low calcification stress observed for the species *T. agglutinans* is suggested to be related to the absence of any physiological impact as observed for the species *T. tenuissima* by van Dijk et al., 2017.

As regards the benthic foraminiferal ecological response to environmental stress, earlier studies showed that foraminiferal diversity and density can be affected by environmental factors (e.g. pH, organic carbon content, oxygenation) (Martins et al., 2016; Sabbatini et al., 2012; Murray, 2006) but also by natural or anthropogenic pollution (e.g. heavy metal; Martins et al., 2013; Frontalini and Coccioni, 2011). The high resistance and high inter/intraspecific variability are related to ecological and physiological mechanisms that allow foraminifera to live and adapt to peculiar stress conditions. In this regard, Dias et al. (2010) showed how foraminifera are sensitive to the ocean acidification in the shallow waters around Ischia and the assemblages shift from calcareous to agglutinated forms in relation to pH decrease. Also, Frontalini et al. (2018) documented how cytological and ultrastructure alteration of foraminifera, caused by heavy metal toxicity, might represent defence mechanisms against stress conditions.

Finally, as for the species ecology, over recent decades, many studies have demonstrated the relationships between foraminifera and prokaryota (i.e. Mojtahid et al., 2011; Nomaki et al., 2006; Bernhard et al., 2018). In this regard, Heeger, 1990 and Lee et al. (1991) demonstrated that foraminiferal species as *R. globularis* and *Quinqueloculina* spp. have prokaryotes in their digestive vacuoles. Some of these associations, that appear to be stable and consistent, are considered symbioses, while others are related to nutritional mechanisms. We may, therefore, hypothesize that the stressful conditions due to cigarette butt leachate might cause 1) prokaryote death by decreasing foraminiferal energy sources and calcification rate (Hallock, 1999); 2) increase on symbionts division rate and overpopulation of the foraminiferal cytoplasm, killing them. In our study, the first hypothesis is more probable than the other, in fact after the experiment a cytoplasm bleaching was observed, and this could have been caused by the absence of symbioses.

Concluding, we hypothesize that foraminiferal death during the experiment is not related to decalcification but the decalcifying effect, induced by toxic cigarette butt leachate, may also be the consequence of the increased energy requirements to maintain the same calcification rate under low pH conditions, and this added to the toxicity of other dissolved substances and the death of prokaryote symbiosis may eventually lead to death. In this regard, the FTIR analyses support this hypothesis as we observed.

Obviously, the LC₅₀ experiment simply indicates the high toxicity of cigarette butts at high concentration. In order to tackle the issue and understand how nicotine and other toxic compounds operate on a physiological level or on the biomineralization, future, more detailed analyses are needed.

5. Conclusions

The leachates of smoke cigarette butts affect the vitality and the test building mechanism of the perforate calcareous *R. globularis*, the imperforate calcareous *Quinqueloculina* spp. and the agglutinated foraminifera *T. agglutinans*. In particular, starting from 4 cigarette butts/L,

the decalcification process for all species considered is observed and the death of individuals for both calcareous specimens belonging to *R. globularis* and *Quinqueloculina* spp. Furthermore, for *R. globularis* at the highest concentration (16 cigarette butts/L), the curve reaches zero. Instead, the agglutinated species shows a different trend and appears more resistant to leachate toxicity. Thanks to FTIR analysis we hypothesize that the two processes: foraminiferal death and decalcification are not related but both could depend on the pH reduction measured in the cigarette butts leachate and on the toxicity of other dissolved substances, in particular nicotine, which lead to physiology alteration and in many cases cellular death.

Furthermore, these LC₅₀ results suggest that 1) foraminifera, regardless to the shells structure, are more resistant than other aquatic organisms; 2) the leachate of human-smoked cigarette butts appears to be more toxic compared to leachate of artificially-smoked cigarette butts (with or without filters); 3) the leachate appears to have an acidifying effect on the solution, causing possibly damage to the marine bio calcifying organisms.

To conclude, the litter cigarette butts might represent a real threat for aquatic environments and further studies are highly needed to better understand the effects on environmental quality and associated ecological risks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2020.105150>.

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