Genotoxicity of citrus wastewater in prokaryotic and eukaryotic cells and efficiency of heterogeneous photocatalysis by TiO₂

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A B S T R A C T
The presence of (±)-α-pinene, (+)-β-pinene, (+)-β-caryophyllene, and R-(+)-limonene terpenes in wastewater of a citrus transformation factory was detected and analyzed, in a previous study, by using Solid Phase Micro-extraction (SPME) followed by GC analyses. Purpose of that research was to compare the genotoxic responses of mixtures of terpenes with the genotoxicity of the individual compounds, and the biological effects of actual wastewater. Genotoxicity was evaluated in the Salmonella reversion assay (Ames test) and in V79 cells by Comet assay. Ames tests indicated that the four single terpenes did not induce an increase of revertants frequency. On the contrary, the mixtures of terpenes caused, in the presence of metabolic activation, a highly significant increase of the revertants in TA100 strain in comparison to the control. The Comet assay showed a significant increase in DNA damage in V79 cells treated for 1 h with single or mixed terpenes. Moreover, the actual wastewater was found highly genotoxic in bacterial and mammalian cells. Photocatalytic tests completely photodegraded the pollutants present in aqueous wastewater and the initial high genotoxicity of samples of wastewater collected during the photocatalytic run, was completely lost in 3 h of irradiation.

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1. Introduction
The cultivation of oranges is an important industry and a significant component of the economy of Mediterranean countries. Citrus wastewater derives from the production of citrus juice and the extraction of essential oils as byproduct. This type of effluent is characterized by very high organic loads (BOD: 20–1400 mg/l; COD: 100–2000 mg/l), by high variability of low values of pH, by the presence of low concentrations of nutrients (especially nitrogen and phosphorus) and flavonoids and heteropolysaccharides, as for instance hesperidin and pectin, in colloidal form. Another peculiarity of the citrus effluent is the presence of essential oils and terpenes. The mixture of four terpenes, but not the single terpenes, induced gene mutations in V79 Hamster Chinese cells [11]. The terpenes are secondary metabolites produced from carbon photosynthesis and are involved in the attraction of pollinators and in the defense against insects, herbivores and microbes [5–7]. They are industrially used in foods and in cleaning products. Only few conflicting studies exist [8–10] on the biological effects of essential oils and terpenes.

In a previous paper, we have evaluated the toxicity bioassays on aquatic macroinvertebrates and using biochemical biomarkers and it was classified as toxic [4]. The composition of citrus transformation plant wastewater is highly complex, and the toxicity can be due to the high concentration of organics, including the terpenes, the major constituents of essential oils [4]. The terpenes are secondary metabolites produced from carbon photosynthesis and are involved in the attraction of pollinators and in the defense against insects, herbivores and microbes [5–7]. They are industrially used in foods and in cleaning products. Only few conflicting studies exist [8–10] on the biological effects of essential oils and terpenes. In a previous paper, we have evaluated the toxicity bioassays on aquatic macroinvertebrates and using biochemical biomarkers and it was classified as toxic [4]. The composition of citrus transformation plant wastewater is highly complex, and the toxicity can be due to the high concentration of organics, including the terpenes, the major constituents of essential oils [4].

The presence of (±)-α-pinene, (+)-β-pinene, (+)-β-caryophyllene, and R-(+)-limonene terpenes in wastewater of a citrus transformation factory was detected and analyzed, in a previous study, by using Solid Phase Micro-extraction (SPME) followed by GC analyses. Purpose of that research was to compare the genotoxic responses of mixtures of terpenes with the genotoxicity of the individual compounds, and the biological effects of actual wastewater. Genotoxicity was evaluated in the Salmonella reversion assay (Ames test) and in V79 cells by Comet assay. Ames tests indicated that the four single terpenes did not induce an increase of revertants frequency. On the contrary, the mixtures of terpenes caused, in the presence of metabolic activation, a highly significant increase of the revertants in TA100 strain in comparison to the control. The Comet assay showed a significant increase in DNA damage in V79 cells treated for 1 h with single or mixed terpenes. Moreover, the actual wastewater was found highly genotoxic in bacterial and mammalian cells. Photocatalytic tests completely photodegraded the pollutants present in aqueous wastewater and the initial high genotoxicity of samples of wastewater collected during the photocatalytic run, was completely lost in 3 h of irradiation.

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at concentrations identical to those found in actual effluents were used.

Notably these types of actual effluents contain not only terpenes but also many other compounds and some of them are unknown; moreover, information about their genotoxicity is scarce. For this reason we have also studied the biologic effects in Salmonella typhimurium and in V79 Hamster Chinese cells of the actual aqueous effluents.

The incomplete removal of persistent organic species observed after the conventional wastewater treatment, prompted us to verify, by performing Total Organic Carbon (TOC) analyses, if heterogeneous photocatalysis could be successfully applied as a coupled technology to the purification of this type of actual effluents. This method has been widely used for complete degradation of several organic pollutants [12–14] and in particular for terpenes present in actual effluents [11]. Moreover experiments were carried out to verify if the photocatalytic treatment influences the genotoxicity of the effluents. In this regards Ames tests have been performed by using samples withdrawn at different times during the photocatalytic runs.

2. Materials and methods

2.1. Chemicals

(*,*)-β-pinene, (+)-β-pinene, (+)-3-carene, and R-(+)-limonene were purchased from Sigma–Aldrich. For the biological study fresh solutions were prepared by dissolving each terpene in a bacterial nutrient broth or in a mammalian medium (D-MEM) supplemented with 0.5% DMSO (Sigma), while methanolic solutions were prepared for the analytical study. All standard reagents used, namely, terpinel-4-ol, caryophyllene, neryl acetate, β-citronellol, trans-geraniol, were purchased from Fluka (Buchs, Switzerland). Mix hydrocarbons from C10 to C26, were purchased from Supelco.

2.2. Wastewater samplings

Samples were collected in a citrus fruit transformation factory that utilizes activated sludge treatments to purify wastewater. The types of aqueous effluents studied were: untreated (B) and treated (C) wastewater. Three samples of both B and C effluents were collected at each sampling location during the period of activity of the factory in 2008 and 2010.

2.3. HS-SPME-GC/FID analysis

The combination of HS-SPME-GC/FID [15,16] offers high instrumental sensitivity allowing for the simultaneous identification and quantification of each terpene both in the industrial and synthetic samples. The extraction procedure as the choice of PDMS (polydimethylsiloxane) fiber, adsorption and desorption times [17,18] were the same optimized in 2008 [11].

For each sample three SPME extractions and desorptions were realized. All the analyses of the synthetic solutions and the linear calibration plots (R between 0.993 and 0.999) were performed using stock solutions obtained by diluting the four standards methanolic terpenes solutions (200 ppm) in ultrapure water to concentrations ranging between 5 and 500 ppb (standard deviation <15%), with addition of neryl acetate as the internal standard (IS). The limit of detection (LOD) was fixed between 1 and 3 ppb, i.e. when the signal was three times higher than the noise. Conversely, the limit of quantization (LOQ) was fixed between 3 and 5 ppb, i.e. when the signal was 10 times higher than the noise.

Chromatographic analyses were performed on a Shimadzu GC 2010A equipped with a Supelcowax (CW) column (Supelco) 30 m long, 0.25 mm i.D., 2.5 μm thick and a FID detector. A temperature programme from 60 °C (maintained for 3 min) to 130 °C with 15 °C/min ramp rate and then to 240 °C (maintained for 1 min) was set. The linear velocity of the carrier gas (He, 99.9995%) was fixed at 25.9 cm/s. The FID temperature was set at 250 °C.

2.4. HS-SPME-GC/MS analyses

The sampling by HS-SPME was identical to GC/FID. Analyses were performed using a Thermo Fisher Scientific GC/MS equipment using a Focus GC, coupled with a quadrupole DSQ II mass spectrometer. Separation of compounds was carried out with the same Supelcowax column and gas chromatographic method of GC/FID analysis. The identical linear velocities of 25.9 cm/s was set for GC/FID and GC/MS, while the transfer line temperature was set at 200 °C.

Electron impact mass spectra were recorded at 70 eV ionization energy in the 35.0–450.0 dalton mass range at 2 scan/s; the quadrupole temperature was set at 225 °C.

Collected data were processed with the instrument data system and chromatographic and spectrometric results showed excellent reproducibility (SD ≤ 4%). Each determination was repeated three times.

2.5. Ames tests

The Ames tests were performed by plate incorporation procedure as previously described [19]. S. typhimurium strain TA100 was grown in 20 ml nutrient broth (Oxoid no 2) with ampicillin (0.1 ml of 8 mg/ml ampicillin solution, Sigma–Aldrich) in a shaking incubator at 37 °C until the suspension reached a density of about 10^9 cells/ml (measured as absorbance at 650 nm). This suspension (100 μl) was added to 2 ml molten overlay agar (supplemented with 18% glucose and 0.2 ml of 0.5 mM histidine/biotin solution) together with aqueous solutions of the single terpenes or the mixture of four terpenes in the absence or in the presence of 500 μl S9 (10%) mix and poured onto minimal-agar petri dishes. The solutions, prepared separately for each terpene at the concentrations corresponding to those present in the wastewater B (untreated) and C (treated) sampled in 2008 [11], were used individually or concurrently. The inverted plates were held at 37 °C in a dark incubator for 48 h and then the revertant colonies were scored.

The strain was tested without addition of foreign compounds as the negative control and with 1 μg/plate methyl methane sulphonate (MMS, CAS 66273 Sigma–Aldrich) as the positive control. Moreover, increasing doses (0, 10, 20, 50, and 100 μl/plate) of actual wastewater B or C (sampled in 2010), filtered by passing through 0.45 μm filters, were tested with the same procedure above described. Each determination was carried out in triplicate and two independent experiments were carried out at least.

Samples (100 μl/plate) collected every hour during the photocatalytic degradation of wastewater C were tested with the same procedure.

For metabolic activation, S9 mix was prepared on ice immediately before its usage by adding solutions of phosphate buffer at pH 7.4, NADP, G-6-P, KCl and MgCl_2 up to final concentrations of 0.1 M, 4, 6, 8, 33 mM, respectively. Subsequently 10% rat liver S9 (Araclor-1254 induced, Trinova Biochem.) was added.

A sample was considered mutagenic when an increase of the number of revertants up to the double with respect to the negative control was reached. The number of spontaneous revertants ranged between 100 and 200 for strain TA100, under the experimental conditions used.
2.6. Cell line

V79 Chinese hamster cells were cultured in D-MEM (Gibco, Invitrogen), supplemented with 5% foetal calf serum (Invitrogen), penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained at 37 °C in a 5% CO₂ humidified incubator.

2.7. Viability

Viability of V79 Chinese hamster cells was evaluated after 1 h treatment both in the presence of synthetic solutions containing the single terpenes, used individually or concurrently, or in the presence of actual effluents filtered by passing through 0.45 μm filters, with trypan blue dye. This dye is normally taken up by non-viable cells but not by viable cells because it only penetrates through membranes of damaged cells. Treated and untreated cells were stained for 5 min with 0.4% trypan blue (Sigma–Aldrich), and counted using a Burker chamber under a light microscope with a 20× magnification. Cell viability was expressed as the percentage of viable cells compared to the total number of cells counted.

2.8. Comet assay

The alkaline single cell gel electrophoresis (SCGE) assay, also known as Comet assay, was carried out according to the protocol of Singh and McCoy [20]. After 24 h of growth, V79 Chinese hamster cells were exposed for 1 h to the single terpene or concurrently to the four terpenes at concentrations corresponding to those found in actual wastewater B (untreated) and C (treated) sampled in 2008 [11] or to 100 μl/plate of actual wastewater B, and C sampled in 2010 and filtered by passing through 0.45 μm filters. After exposure, the cells were washed twice with Hanks’ salt solution and collected by trypsinization. Because the viability of cells after treatment, determined by trypan blue dye, was always >70% [21], the cells were immediately processed in the SCGE assay. They were centrifuged at 800 rpm for 10 min, re-suspended in PBS (2 × 10⁵ cells/10 μl final concentration) and mixed with 65 μl of 0.7% Low Melting Agarose (LMA, Bio-Rad). Subsequently they were layered on a slide (GeBond film, Sigma), previously coated with a layer of 0.5% Normal Melting Agarose (NMA, Fisher Molecular Biology), and covered with another layer of LMA. The agarose suspension was covered with a coverslip and placed at 4 °C for 10 min. The coverslip was gently removed and the slide was submerged into lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10.0) for 1 h under dark at 4 °C. After lysis the slides were placed for 15 min in a horizontal electrophoresis gel tray containing fresh alkaline buffer (300 mM NaOH, 1 mM Na₂EDTA, pH >8) and then subjected to an electric field of 0.7 V/cm in the dark at 4 °C for 20 min. Following electrophoresis the microgels were neutralized in 0.4 M Tris–HCl (pH 7.5), dehydrated in methanol for 2 min and allowed to dry at room temperature. The DNA was stained with ethidium bromide (2 μg/ml) (Electran BDH), and visually examined by fluorescence microscope (Nikon Microphot-FXA/SA), equipped with a HBO 100 mercury lamp and a suitable filter. Photomicrographs were processed using Photoshop 6.0 Software (Adobe). For qualitative evaluation, at least 1000 cells were scored for each dose. Cells were classified as undamaged, damaged with Comet shape and ghosts with small head and large tails. DNA damage was quantified by tail length (L Tail) and Tail Moment (TM) values calculated by Comet Assay Software Project (CASP). The L Tail is the distance from the middle of nucleoid core to the end of the tail; TM is defined as the product of the percentage of DNA in the tail distribution and L Tail.

Data derived from three independent experiments, with at least 50 cells randomly scored per experiment (from two replicate slides each), were processed with the Kruskal–Wallis non-parametric statistical method.

2.9. Photocatalytic experiments

The photocatalytic experiments were carried out on treated (C) wastewater collected on 2010. Before the experiments the wastewater was filtered with 0.20 μm filters (Millex, Millipore) and in order to remove the inorganic carbon, concentrated H₂SO₄ was added up to reach a pH of 4.5. The photocatalytic runs were performed in a 0.15 l Pyrex cylindrical batch photoreactor with an immersed lamp, continuously bubbling oxygen in the suspension and using an amount of TiO₂ Degussa P25 (ca. 80% anatase; 20% rutile, BET specific surface area: ca. 50 m² g⁻¹) equal to 0.4 g l⁻¹. Before starting the irradiation, the reacting suspension was magnetically mixed for ca. 30 min to achieve the adsorption/desorption equilibrium of the species contained into the solution. The suspensions were magnetically stirred throughout the runs and irradiated by a medium pressure Hg 125 W lamp (Helios Italquartz, Milano). The irradiance reaching the reacting suspension, measured in the wavelength range 320–390 nm with a UVX Digital radiometer, was ca. 10 mW cm⁻². Due to the complexity of the solutions, only the total dissolved organic carbon (TOC) was analyzed. The analyses were performed at fixed intervals of time by using a Shimadzu 5000A instrument (error: ±1 ppm), after separation of the photocatalyst from the suspension. The samples withdrawn at different time during the photocatalytic experiments were also subjected to Ames test.

3. Results and discussion

3.1. Biological effects of exposure of bacterial and mammalian cells to single terpenes or to the mixture of four terpenes

3.1.1. Ames tests

Ames tests with S. typhimurium strain TA100 were used to assess the mutagenic activity of single terpenes, i.e. (±)α-pinene, (±)β-pinene, (±)3-carene, and R-(±)limonene, or of the mixture of the four terpenes in the presence or absence of S9 mix. For this purpose, synthetic solutions of terpenes at the concentrations corresponding to those found in the wastewater B (untreated) and C (treated) sampled in 2008 [11] were utilized (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Concentrations (μM) of terpenes found in samples B and C derived from the citrus transformation factory (2008).</th>
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<tr>
<td>(±)α-Pinene</td>
</tr>
<tr>
<td>B</td>
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<tr>
<td>C</td>
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(B) Untreated and (C) treated wastewater.

As shown in Fig. 1, each terpene was not mutagenic in the presence and absence of S9 mix; indeed the number of revertants per plate was very close to the 100–200/10⁸ spontaneous revertants of strain TA100, according to the reports available on the investigation of the genotoxicity of such compounds individually. In fact, racemic mixtures of α-pinene, such as (+) and (−) enantiomers of α-pinene [22], or R-(±)limonene [23] have been previously shown to be not mutagenic in the Salmonella mutagenicity assay; no previous tests of (+)3-carene or (±)β-pinene have been reported in the literature. The negative results in the Ames test were consistent with our previous findings in V79 cells; in fact the exposure at a single terpene did not induce gene mutation [11].

Moreover we exposed concurrently S. typhimurium to synthetic solutions of the four terpenes in concentrations comparable to those found in the wastewater sampled in 2008 to study the cumulative effect, if any. Interestingly, the mixture of terpenes in the
presence of S9 mix, induced a reproducible increase in the number of revertant colonies per plate; in particular, the increase induced by concurrent presence of synthetic solutions of terpenes at the concentrations corresponding to those found in wastewater B (untreated) and C (treated) sampled in 2008 (Table 1), induced doses related DNA damage. In fact, DNA damage induced by single terpenes at concentrations corresponding to B (untreated wastewater) was significantly different (p < 0.001) in comparison to the control (Fig. 2) and was higher than the damage induced by single terpenes at concentrations corresponding to C (treated wastewater). However, the level of damage induced by the mixture of terpenes was significantly higher (p < 0.001) compared to the single compounds. This result confirms that the biological effects of mixture of chemicals could be different from that of the single compounds and suggests that mutagenic metabolite/s can originate from the mixture during the phase I of the metabolism.

### 3.1.2. Comet assay

To evaluate the damage induced in V79 cells by exposure for 1 h to a single terpene or to a mixture of them, we used the Comet assay. Preliminary qualitative analyses, performed by classifying cells in three groups: undamaged-, damaged- and ghost-nucleus (Fig. 3), indicated that each terpene, at concentrations corresponding to those revealed in actual effluents B (a) and C (b) in the absence or in the presence of S9 mix. Control, i.e. untreated bacteria. MMS: positive control, i.e. bacteria treated with methyl methane sulfonate (1 μg/plate). Results from three experiments ±S.D. are presented.

Fig. 1. Number of revertants/plate obtained after exposure to aqueous solutions of the single terpenes at concentration corresponding to those revealed in actual effluents B (a) and C (b) in the absence or in the presence of S9 mix. Control, i.e. untreated bacteria. MMS: positive control, i.e. bacteria treated with methyl methane sulfonate (1 μg/plate). Results from three experiments ±S.D. are presented.

Fig. 2. Number of revertants/plate obtained after exposure to aqueous solutions of the mixture of the four terpenes at concentrations corresponding to those revealed in actual effluents B and C in the absence or in the presence of S9 mix. Control, i.e. untreated bacteria. MMS: positive control, i.e. bacteria treated with methyl methane sulfonate (1 μg/plate). Results from three experiments ±S.D. are presented.

Fig. 3. Representative photomicrographs of undamaged- (a), damaged- (b) and ghost-nucleus taken with a fluorescence microscope at 100× magnification.

Fig. 4. Percentage of cells with undamaged-, damaged- and ghost-nucleus after exposure to aqueous solutions of the single terpenes or mixture of the four terpenes at concentrations corresponding to those revealed in actual effluents B (a) and C (b). Control, i.e. V79 Hamster Chinese cells untreated. Results from three experiments ±S.D are presented. * p < 0.001 vs. control, according to the Student’s t test.
of DNA damage induced by terpenes at concentrations of sample C was higher than control values which were within historical records in our laboratory (Fig. 4b). Further, the frequency of damaged nuclei differed for each terpene tested.

The responses generated by concurrent exposure of cells to the four terpenes, at concentrations corresponding to B or C, confirmed that the frequency of damaged cells was significantly different with respect to the control, but it was lower than the sum of frequency of damaged cells induced by the single terpenes (Fig. 4a and b).

Quantitative evaluations, performed after treatment with single terpenes or mixture of the four terpenes at the concentrations corresponding to B or C, revealed a highly significant (p < 0.0001) increase of the L Tail and TM in comparison to the control (Fig. 5a and b).

Results from both the qualitative and the quantitative analyses demonstrated, for the first time, that (±)-α-pinene, (+)-β-pinene, (+)-3-carene, and R-(+)-limonene are capable to cause damage to DNA. Moreover, it is worth to notice that the average of L Tail values induced by the mixture of the four terpenes was lower than the sum of the average of L Tail values induced by the single terpenes. This result suggests that the concurrent presence of the four terpenes could induce the formation of cross-links; in fact, it is known that the presence of crosslinks would hamper the DNA breakage by the Comet [24].

3.2. Biological effects of exposure of bacterial and mammalian cells to actual wastewater

As scarce information is available about Citrus processing wastewater toxicity [4], we have also studied the biologic effects in bacterial and mammalian cells of the actual effluents.

Results obtained by exposure of S. typhimurium cells to 10, 20, 50, and 100 μl/plate of actual wastewater indicated that 50 and 100 μl of wastewater B and 100 μl of wastewater C induced a highly significant (p < 0.0001) number of revertants with comparison to the control (Fig. 6).

The DNA damage induced by these effluents to mammalian cells was indicated by results obtained from Comet assay. The qualitative analyses indicated that 45% and 30% of V79 cells, treated for 1 h with 100 μl of actual wastewater B or C, respectively, were damaged (Fig. 7a). In addition significant increases (p < 0.0001) in L Tail and TM were detected (Fig. 7b). It is evident that the actual effluents were genotoxic for both the biological systems.
3.3. HS-SPME-GC/MS

Gas chromatographic (GC) methods have been extensively applied in this field, in connection often with mass spectrometric (MS) analysis, to detect by the headspace method the composition of the volatile components of water and wastewater [25,26]. Linear retention indices (LRI) were calculated using Kovats’ equation [27] with the sequence of linear hydrocarbons from C10 to C26. HS-SPME-GC/FID analyses of microfiltered wastewater C (treated) indicated that the concentrations of the four terpenes were below the limits of determination. Preliminary qualitative analysis by HS-SPME-GC/MS showed that other volatile compounds, as eucalyptol, citronellol and trans-geraniol, were present (Fig. 8). It has been reported that the non-cyclic terpene alcohols citronellol and trans-geraniol are inactive in bacterial and mammalian cell systems [28] while the literature data on mutagenic and genotoxic effects of eucalyptol are controversial [29].

Volatile compounds of microfiltered wastewater C were identified first by a critical and reasoned comparison with mass spectral data within the NIST 2005 library. Subsequently compounds listed were verified on the LRI list. In addition, compounds were compared to their related standards.

Of course, in addition to the volatile compounds detected, it cannot be excluded the presence of other soluble substances that are responsible of the genotoxicity of wastewater and notably traces of other molecules as for instance, Copaene, α-Bergamotene, Caryophyllene and Valencene (all sesquiterpenes), were found in unfiltered wastewater C. It has been reported that sesquiterpenes are present in citrus essential oil [30,31]; no cytotoxicity or mutagenicity in a large range of concentrations was observed by Gonçalves et al. [32], but the authors did not exclude the possibility that the bioactivity of the chemicals in a mixture could be different from that of the isolated compounds. Moreover cytotoxic and genotoxic activity of P. gaudichaudianum essential oil has been attributed to α-humulene and (E)-caryophyllene sesquiterpenes [33]; on the contrary, anticlastogenic activity of β-caryophyllene has been reported [34].

3.4. Photocatalytic experiments

The photocatalytic technology represents an advanced oxidation technology suitable to degrade almost all types of organic pollutants dissolved in water. The most used photocatalyst is polycrystalline TiO2 in the anatase form, due to its low cost, insolubility in water and nontoxicity. When aqueous suspensions of this semiconductor are irradiated, at the solid–liquid interface a great variety of photoinduced chemical reactions, able to degrade the organic molecules present, can occur by means of formation of very reactive radical species that are generated in the presence of O2 and H2O. Reactions (1)–(7) show some events that can occur after the photoproduction of electrons and holes in the conduction (CB) and valence band (VB), respectively:

\[
\text{TiO}_2 + h\nu \rightarrow \text{TiO}_2 \left( e_{\text{CB}}^0 + h_{\text{VB}}^0 \right) \\
\text{OH}^+ + h_{\text{VB}}^0 \rightarrow \cdot \text{OH} \\
\text{O}_2 + e_{\text{CB}}^0 \rightarrow \cdot \text{O}_2 \\
\cdot \text{O}_2^- + \text{H}^+ \rightarrow \cdot \text{HO}_2 \\
2 \cdot \text{HO}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + \cdot \text{O}_2 \rightarrow \cdot \text{OH}^- + \cdot \text{OH} + \text{O}_2 \\
\text{Terpene + radical species (for instance } \cdot \text{OH} + \cdot \text{O}_2^- \rightarrow \text{H}_2\text{O} + \text{CO}_2 \)
\]

Some preliminary blank tests indicated that no reactivity occurs in runs performed under the same experimental conditions used for the photo-reactivity experiments but in the absence of catalyst, oxygen or light. It was concluded that the simultaneous presence of O2, catalyst and irradiation is needed for the occurrence of the degradation process of the organic compounds present in the industrial wastewater. The photocatalytic runs were carried out by using the actual wastewater (sample C, treated wastewater). In Fig. 9 the Total Organic Carbon concentration (TOC) vs. irradiation time is reported for a representative run. The disappearance in ca. 5 h of the organic carbon (the residual TOC was ca. 1 mg l\(^{-1}\)) in the experimental error of the instrument) indicated that in this actual situation the photocatalytic technology can be applied for the complete mineralization of the several pollutants present in the treated wastewater (C sample). Results obtained by exposure of S. typhimurium cells to sample C before and during the photocatalytic treatment are also reported in Fig. 9. The samples collected from 0 to 2 h of irradiation showed genotoxic capability: in fact they induced a significant increase of revertants per plate.

[Fig. 8. Chromatograph of microfiltered wastewater C (treated) by HD-SPME-GC/MS analysis.]

[Fig. 9. TOC (\(\circ\)) concentration (ppm) vs. irradiation time (h) during a photocatalytic test of actual wastewater C. Histograms indicate the number of revertants/plate obtained after exposure to sample withdrawn at different irradiation time during the same photocatalytic run. (a) Untreated bacteria; (b) bacteria treated with TiO2.]
The samples, subsequently collected, lose this capability; in fact these samples induced a number of revertants per plate very close to 100–200/10^8 spontaneous revertants of strain TA100. Notably they were completely degraded at the end of the photocatalytic treatment because the number of revertants per plate returned down to the spontaneous background.

4. Conclusion

The composition of citrus transformation plant wastewater is highly complex and the terpenes constitute a relevant organic load. So, initially we have addressed our interest to evaluate the biological effects in bacterial and mammalian cells of (±)-

The single terpenes resulted not mutagenic in S. raphimurium, but they were capable to induce DNA damage in mammalian cells. This result is the first experimental evidence that these terpenes affect the integrity of genome. To-date, we do not know if the DNA damage is the result of direct genotoxicity or it is related to an indirect effect, i.e. the terpenes capability of inducing reactive oxygen species. This is possible by considering that this capability was demonstrated by Singh et al. for α-pinene [35]. The mixture of four terpenes resulted strongly mutagenic in bacterial cells, and it was able to induce DNA damage in mammalian cells also at the lowest concentrations. The response obtained in concurrent treatments could be the result of some sort of interaction between the four terpenes or simply an additive effect.

Both hypotheses are plausible, but they must be corroborated by specifically designed experiments to clarify the joint chemical action of the four monoterpenes (individually considered as safe substances) able to induce DNA damage when they are contemporaneously present.

As a new finding the actual wastewater was found highly genotoxic in bacterial and mammalian cells. This result indicates that the industrial biological treatment is partially effective. On the contrary, the photocatalytic technology was effective for the complete mineralization of the several pollutants present in the treated wastewater. Moreover the samples collected after two hours of irradiation lose genotoxic capability, indicating that the transformation/mineralization of the starting organic compounds is beneficial with respect to the genotoxicity of the wastewater.

Acknowledgment

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References

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As a new finding the actual wastewater was found highly genotoxic in bacterial and mammalian cells. This result indicates that the industrial biological treatment is partially effective. On the contrary, the photocatalytic technology was effective for the complete mineralization of the several pollutants present in the treated wastewater. Moreover the samples collected after two hours of irradiation lose genotoxic capability, indicating that the transformation/mineralization of the starting organic compounds is beneficial with respect to the genotoxicity of the wastewater.
