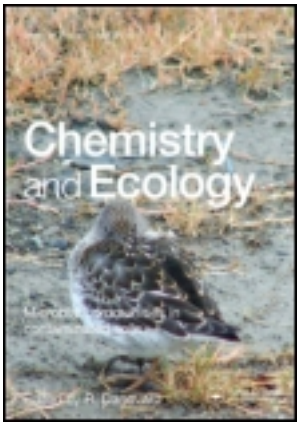

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Effects of arsenic alone and in combination with other pollutants in *Dunaliella tertiolecta*

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Effects of arsenic alone and in combination with other pollutants in *Dunaliella tertiolecta*

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In this article, the effects of As alone and in combination with other pollutants on the flagellate *Dunaliella tertiolecta* were studied. Cultures of this microalga were subjected to growth-inhibition tests, according to the ISO protocol, using the same As concentration range for all tests; the results showed an obvious toxic effect for cultures exposed to As and As combined with Zn, whereas for cultures exposed to As combined with the nonionic surfactant Triton X-100 and ethylene glycol, hormetic effects were also observed. The same acetone extracts of cultures used in the growth-inhibition test were further examined using 3D visible (Vis) and attenuated total reflectance infrared (FTIR-ATR) spectroscopy. 3D Vis spectroscopy showed that As and As mixed with other pollutants did not have common dose–response effects. For example, most chlorophyll and carotenoid pigments were shown to be increased and/or decreased within the same growth-inhibition test. FTIR-ATR examination of the same solutions showed other differentiated effects of As alone and As combined with other pollutants, revealing quantitative modifications of phospholipid and fatty acid contents and structural modification of DNA and RNA. In addition, a multivariate elaboration of FITR spectra, performed by means of a multivariate statistical technique, principal component analysis (PCA), showed that the observed molecular modifications in *D. tertiolecta* can be clustered according to the different types of exposure to pollutants. These findings suggest that the molecular modifications caused by As exposure are also related to the type of toxic mechanism (i.e. synergic or additive) which results from the presence of other pollutants.

Keywords: arsenic; *Dunaliella tertiolecta*; toxicity; hormesis; growth-inhibition test; chlorophyll pigments; carotenoids; visible spectroscopy; infrared spectroscopy

1. Introduction

In the marine environment arsenic arises from hydrothermal and volcanic activities and from industrial discharge. Because of the high bioconcentration capacity of many marine organisms, As can reach humans via the most common food chains, causing premalignant and malignant skin tumors, pregnancy complications and mortality [1–3]. For this reason, related risk-assessment studies require the identification of possible markers to reveal the toxic effects of the presence of As. Several studies have investigated the effects of As on different probe organisms belonging to terrestrial and marine environments, trying to relate the response of the tested organisms to the presence of As. For example, chlorophyll *a* has been shown to be a potential marker for several

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environmental stresses in the marine environment, such as eutrophication [4,5] and metal exposure [6]. The response of chlorophyll and carotenoid pigments to the presence of pollutants is general evidence in terrestrial organisms also; biological monitoring shows a modified chlorophyll *a* to chlorophyll *b* ratio for lichens in the presence of pollutants [7], whereas changes in chlorophyll *a* content have been observed in the presence of heavy metals [8,9] and polycyclic aromatic hydrocarbons [10]. Some studies have pointed out the physiological response of *Dunaliella salina* and *Dunaliella tertiolecta* to Cu and As [11–14] and in addition its high As bioconcentration factor [3], and so we performed a study to describe some specific responses of *D. tertiolecta* to As exposure.

In any case, it should be taken into account that the toxic effect of any pollutant is also related to the presence of other pollutants, because this can lead to marked modifications in the toxic effect and mechanism [15]; therefore, research into specific markers for the presence of As should include its potential additive or synergic effects related to the simultaneous presence of other pollutants. With this aim, conventional growth-inhibition tests were performed on cultures of *D. tertiolecta* exposed to As alone and As combined with other pollutants such as ethylene glycol, nonionic surfactant Triton X-100 and Zn. The algal extracts used in the growth-inhibition tests were also examined using 3D visible (3D Vis) and infrared (FTIR) spectroscopy to detect qualitative and structural molecular modifications caused by exposure to As.

2. Methods and materials

2.1. *Sampling preparation*

2.1.1. *Culture medium for algal growth (synthetic seawater)*

The culture medium for algal growth was prepared according to the ISO protocol [16].

Synthetic seawater was prepared according to the above ISO protocol using analytical reagent grade salts (Carlo Erba Milan, Italy) after filtering the resulting solution on a 0.45 μm Millipore® membrane prior to any growth-inhibition test. Only MilliQ® deionised water was used for synthetic seawater.

2.1.2. *Standard samples of pollutants for ecotoxicological test by* D. tertiolecta

All the pollutants considered in this study were prepared in MilliQ® deionised water with an initial concentration of 1 $g \cdot L^{-1}$, using analytical reagent grade standard samples (Carlo Erba) and arsenate was used as a standard of As. For execution of the growth-inhibition test, solutions were diluted using synthetic seawater.

2.1.3. *Algal inoculum*

A volume of standard sample with pollutant (250 mL) was divided into six 40-mL subsamples (one control and five samples) for execution of the bioassay. The samples were placed in sterilised glass flasks.

An algal suspension sampled during the exponential growth phase was prepared with an initial concentration between 1.2×10^6 and 1.0×10^6 cells$\cdot mL^{-1}$, as measured using a Coulter Automatic Counter (Beckman). This starting value is fundamental in *D. tertiolecta* cultures to warrant linear relationships between Vis absorption and cell density so that the pigment measurements may also be accurate [13].

A 0.5-mL aliquot (initial concentration 1–1.2×10^6 cells$\cdot mL^{-1}$) was added to each sample using an analytical microsyringe.

Glass flasks (five polluted samples and a control sample) were placed in a thermostatic chamber ($20 \pm 2\,^{\circ}C$) and illuminated by a cool white light source in the range 7000–8000 lux for 72 h. Any inhibition test was performed in duplicate to reduce the effects of potential errors in sample handling.

At the end of the test, the concentration of living cells was measured using the above-mentioned automatic counter.

2.1.4. *Vis spectroscopic studies of algal cultures*

The remaining water samples with algal cultures were used to extract pigments according to standard methods for phytoplankton analysis [17,18] and Vis spectroscopic measurements.

Each sample was filtered on a 0.45 μm Millipore® membrane under vacuum, the filter was then placed in a sterilised plastic container and dissolved in 5 mL of an acetone water solution (98 v/v). Dissolution of the filter was supported by mechanical shaking and the supernatant was centrifuged at at 3500 rpm and $\sim 5\,^{\circ}C$ for 20 min. Samples were stored at $-25\,^{\circ}C$ until any spectroscopic measurements. Vis spectra were collected using a Lambda 40 double-beam Perkin–Elmer spectrophotometer by means of a 1 cm pathlength cell with acetone as the spectrophotometric blank.

Spectra were saved as ASCII files for any spectral elaboration. Pigment determinations were performed according to the method described by Eijcklhoff and Dekker [18].

2.1.5. *FTIR studies of algal cultures*

For this study, the same solutions as in the Vis studies were used. FTIR spectra of algal cultures were collected by a single beam Jasco spectrophotometer Mod 410 in attenuated total reflectance (ATR) mode, using a ZnSe crystal with 10 internal reflections. A 0.1-mL aliquot of the acetone solution was placed on the ZnSe crystal and spectral acquisition was performed after evaporation of the acetone. Spectra were collected after 250 scans at $4\,cm^{-1}$ of resolution and saved as ASCII files for any spectral elaboration. Spectra were baseline corrected and an 11-point smoothing filter [19] was applied for noise reduction; the FTIR spectra were then normalised using the intense band at $1745\,cm^{-1}$. The accurate assignment and identification of infrared bands of *D. tertiolecta* has been reported previously [14], but in any case, it was supported by determining the second derivative of the spectra, which is a simple and accurate method for enhancing peak resolution and identification [19,20].

2.1.6. *Elaboration of spectral data*

Two spreadsheets (one for Vis and one for FTIR spectra) were constructed for each growth-inhibition test and then saved as ASCII files. PCA was performed according to the so-called singe value decomposition method [20,21]; with this aim, all ASCII FTIR spectral data were combined. Homemade routines written in Matlab 5.1 (MathWorks, Natik, MA) were used for all the execution of 3D Vis and PCA of FTIR spectra.

3. Results and discussion

3.1. *Results of ecotoxicity test*

The results of the ecotoxicity test are reported in Table 1. EC_{50} values for As and ethylene glycol alone are $1.1\,mg{\cdot}L^{-1}$ and $56\,g{\cdot}L^{-1}$, respectively. When present in the same mixture, the resulting

Table 1. EC_{50} values for pollutants and pollutant mixtures from the inhibition growth test using *Dunaliella tertiolecta*.

Pollutants	Concentration range in the inhibition growth test	EC_{50}	T.U.
As	0.1–2 mg·L^{-1}	1.1 mg·L^{-1}	
Zn	1–30 mg·L^{-1}	10.5 mg·L^{-1}	
Triton X-100	1–25 mg·L^{-1}	10.5 mg·L^{-1}	
Ethylene glycol	5 - 100 g·L^{-1}	56 g·L^{-1}	
As and Zn	As 0.1–1.5 mg·L^{-1} Zn 1–20 mg·L^{-1}	0.5 mg·L^{-1}; 6 mg·L^{-1}	1.02
As and Triton X-100*	As 0.1–1.5 mg·L^{-1}, Triton X-100 1–15 mg·L^{-1}	0.4 mg·L^{-1}; 5.7 mg·L^{-1}	0.9
As and ethylene glycol*	As 0.1–1.5 mg·L^{-1}, Ethylene glycol 1–90 g·L^{-1}	0.8 mg·L^{-1}; 8 g·L^{-1}	0.84

Note: *Indicates the test in which the hormetic effect was observed at the lowest dilution. The range of As was 0.1–2 mg·L^{-1} for all tests, whereas for other pollutants the ranges were 10–80 mg·L^{-1} for ethylene glycol, 2–20 mg·L^{-1} for Triton X-100 and 2–20 mg·L^{-1} for Zn.

EC_{50} value corresponds to a mixture of 0.8 mg·L^{-1} As and 8 g·L^{-1} ethylene glycol. The related toxicity expressed as toxic units (TU) is 0.84, a value very close to the boundary value (i.e. 0.8) between the additive and the synergic mechanism of toxicity [15], which suggests that the two toxic mechanisms might coexist for this type of mixture. This results can be also considered as analogous to the synergic toxic mechanism observed for polyethylene glycol surfactants when used jointly to As herbicide [22].

The EC_{50} value of Triton X-100 is 10.5 mg·L^{-1}. When mixed with As, the observed EC_{50} value corresponds to a 0.4 mg·L^{-1} solution of As and a 5.7 mg·L^{-1} solution of Triton X-100; the corresponding TU value is 0.90, typical of an additive toxic mechanism [15].

The EC_{50} value for Zn is 10.5 mg·L^{-1}. When mixed with As, the observed EC_{50} value corresponds to a 0.5 mg·L^{-1} solution of As and a 6 mg·L^{-1} solution of Zn, with a TU value of 1.02. This is typical of an additive toxic mechanism, here supported by the absence of possible chemical interactions between As and Zn. In fact, the net zero effect of interactions between pollutants (i.e. the case of two cations without chemical interaction) determines the concentration additive toxicity mechanism observed for reactive chemical mixtures [15].

As far as the hormetic effect was concerned, it has been also observed for mixtures of As with ethylene glycol and with Triton X-100 at the lowest concentration tested (Figure 1, lower plot). As is recognised as having potential hormetic power [23] depending reasonably on the characteristics of arsenate to be similar to and then an antagonist of phosphate in the role of supporting plant growth [24–27]. In fact, confirmation of this antagonist role in the presence of phosphate has been observed already for the accumulation of As in *Dunaliella* sp. [28].

However, the reasons for the observed hormetic effect in cultures exposed to As combined with Triton X-100 and ethylene glycol, and absent in cultures exposed to As alone, are unclear and deserve a separate study which is beyond the scope of this article.

3.2. *Visible spectroscopic study of* **D. tertiolecta** *cultures*

3.2.1. *Ordinary Vis spectra*

Figure 1 gives the Vis spectra of acetone extracts of *D. tertiolecta* cultures simultaneously exposed to As and Triton X-100. The strong overlapping peaks of chlorophylls *a* and *b* with carotenoid and pheaophytine contents between 480 and 400 nm do not allow the examination of possible

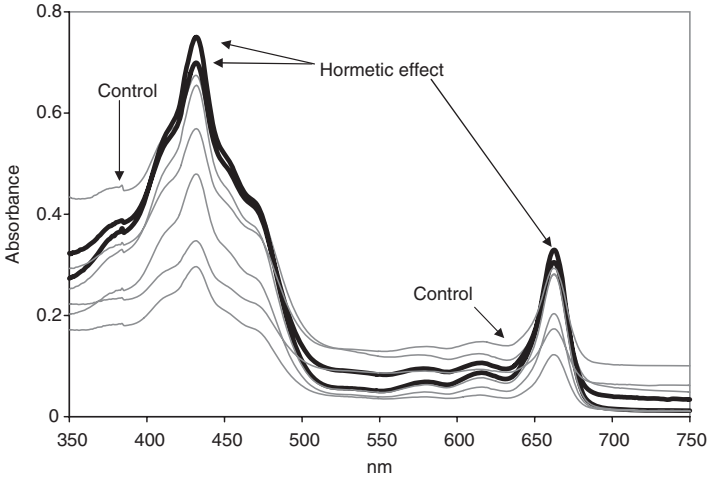

Figure 1. Vis spectra of *D. tertiolecta* culture extracts obtained by the growth- inhibition test with As and Triton X-100 (lower). The two highest black spectra, more intense than the highest grey spectrum (i.e. the control sample) are the evidence of the hormetic effect confirmed by the cell number test.

modifications to the relative ratios, which recent studies have shown to be potential markers of pollution events [5,9]. A re-examination of the above Vis spectra by means of their second-order derivative spectra, not reported here for the sake of brevity, allows a better resolution of the overlapping peaks of pigments showing also that *D. tertiolecta*, like most Chlorophyceae, lacks chlorophyll *c* [29].

In any case, quantitative information related to changes in the pigment concentration ratio, which are generally markers of environmental stress [8,9], remains unclear. The unique observed differences are related the decrease and/or increase in molecular absorptions, dependent on the change in cell number determined by toxic or hormetic effects, and it is not possible to perform a differentiation among the effects of different pollutants on the pigments of cultures.

3.2.2. *3D Vis spectra and specific plots of pigment contents from Vis spectra*

Re-examination of the Vis spectra using 3D plots shows that changes in pigment content are more evident. Using the 3D Vis plots (Figure 2) it is easy to verify that an amount of each pigment may be involved in several changes (either increases or decreases) during each growth-inhibition test, changes which are obviously not observed in the 3D spectra of *D. tertiolecta* cultures obtained by simple dilution of the control sample (Figure 2, left upper plot).

So the evidence from 3D Vis spectra suggests re-examining all the pigment content determined by Vis spectroscopy in accordance with several articles which have addressed the effects of metal pollutants on the pigment content of terrestrial and marine vegetal organisms exposed to As alone and As in mixtures with other pollutants [9,11,28,30,31–33].

For example, modification of the pigment-to-cell number ratios observed in the presence of As are not comparable with modifications encountered in the presence of As combined with other pollutants (Figures S1–S4 – available online only). In fact, any ratio plot is different from another, showing that the chlorophyll *a* to cell number ratio for example, increases in the presence of As (Figure S1, upper plot – available online only), whereas in the presence of As combined with Triton X-100 the ratio values show both increases and decreases (Figure S2, upper plot – available online only).

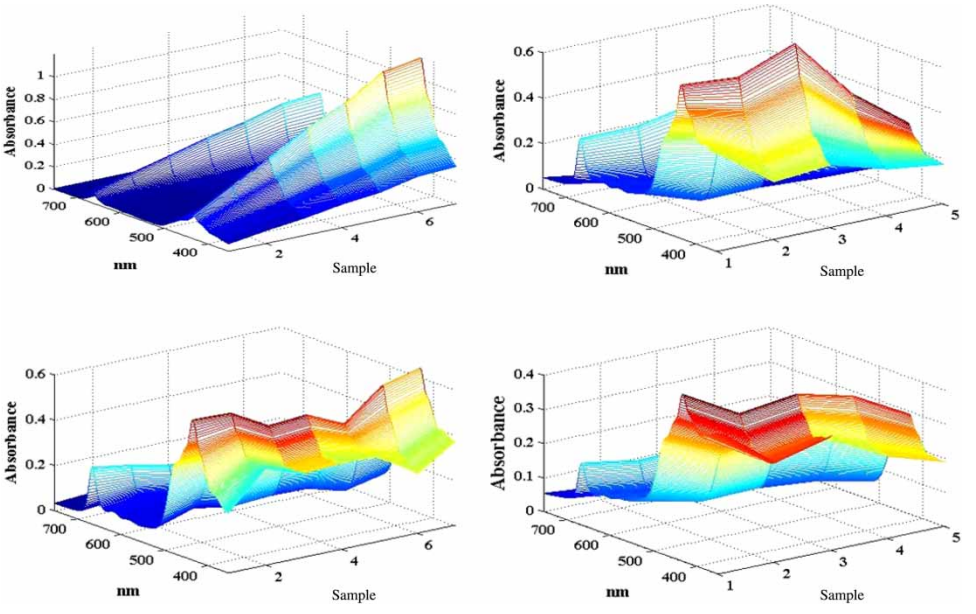

Figure 2. Comparison of 3D Vis spectra of *D. tertiolecta* cultures subjected to the growth-inhibition test and control cultures; (Upper left) Dilution of a control culture; (upper right) As-exposed cultures; (lower left) As with Triton X-100-exposed cultures; (lower right) As with Zn-exposed cultures.

In the same test of cultures exposed to As only, the carotenoid content to cell number ratio is almost constant in the case of exposure to As only (Figure S1, bottom plot – available online only) and tends to increase in the presence of higher levels of As, close to the total inhibition growth effect. This feature of *D. tertiolecta* has been also observed for *Dunaliella* sp. [32] and depends on a protective antioxidant mechanism against the oxidative stress caused by metals [9]. The carotenoid to cell number ratio is different and not comparable in the case of As combined with Triton X-100 or As combined with ethylene glycol (Figures S2 and S3, bottom plots – available online only).

At the highest concentration of each pollutant, close to the full growth inhibition of the cultures, the chlorophyll *a*, *b* and carotenoid to cell number ratios tend to the highest values, although these values are not comparable among them.

The phaeophytin to cell number ratios do not show comparable trends either. In the case of exposure to As with Zn, a comparable trend is observed for chlorophyll *b* and phaeophytin to cell number ratios, showing a direct relationship between degradation of chlorophyll *b* and phaeophytin formation (Figure S4 – available online only). By contrast, no comparable trend is observed for the other pollutants; this suggests that the degradation of chlorophyll *a* and *b* could give other products in addition to phaeophytin (Figures S2–S4 – available online only).

3.3. *FTIR studies*

3.3.1. *General characteristics of* D. tertiolecta *culture FTIR spectra*

Although Vis spectra give information concerning changes in pigment content in the acetone solutions from growth-inhibition tests, FTIR spectra are more powerful in describing the biomolecules present (Table 2); this may elucidate other molecular modifications caused by exposure to different pollutants not visible by Vis spectra. Here, we discuss the criteria applied for band assignment.

Table 2. Table of FT-IR band assignment for spectra of *Dunaliella tertiolecta*.

cm^{-1}	Group	Biomolecules
3460	OH stretching	Carbohydrates, proteins, lipids (sterols and fatty acids), nucleic acids
3020–3035	CH stretching	Unsaturated lipid structures
2950	CH3	Stretching lipids and proteins
2850	CH2	Stretching lipids and proteins
1745	C=O	Stretching lipids (esters of fatty acids)and carbonyl groups of chlorophyll pigments
1710	C=O	Stretching lipids (fatty acids), DNA and RNA
1670	C=C	Alkene groups of chlorophyll pigments
1650	C=O	Stretching proteins (Amide I band)
1540	C−N	Stretching proteins (Amide II band)
1440–1380	CH bending	Alchyl groups
1275	C−O−H	Carbohydrates, proteins, DNA and RNA
1240	P=O	Phospholipids and nucleic acids
1120–1160	C-O-C	Carbohydrates as polysaccharides
1020–1085	P=O	Phospholipids, DNA and RNA
830	C=N (rings)	Nucleotides and chlorophyll pigments
697–753	CH_2 bending	Carbohydrates, proteins, lipids (sterols and fatty acids)

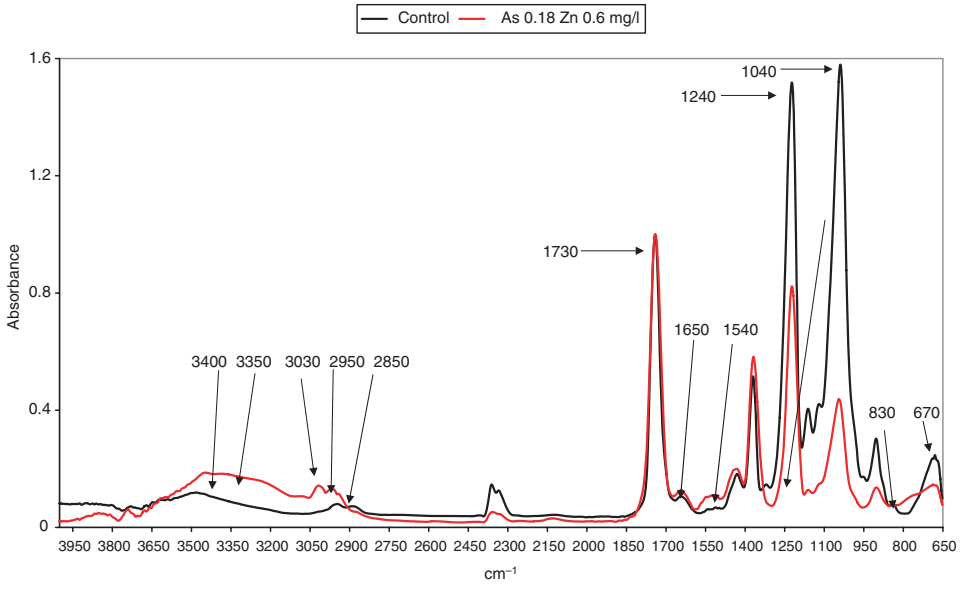

Figure 3. Example of FTIR spectra of control (black) cultures and cultures exposed to As and Zn (red), showing the molecular modifications caused by pollutants (colour online).

The intense band observed at 1745 cm^{-1} (Figure 3) is often used to normalise the spectra coming from cell cultures [14]. Here, this band can be assigned simultaneously to the −C=O group of ketone, the methyl ester group of chlorophyll pigments and the −C=O group of ester fatty acids present in the lipid fraction of the cell membrane. Another band belonging to a −C=O group is observed at 1645 cm^{-1} and this is assigned to the C=O peptide group of proteins (i.e. the so called Amide I band) [34]. Assignment of this band to a peptide group is confirmed by the simultaneous presence of bands between 1540 and 1545 cm^{-1} (stretching vibration of the peptidic −C-N group) and 1280 cm^{-1} (bending vibration of the same −C-N group); these bands are called Amide II and Amide III, respectively [34]. The peptide bands arise from the protein content of the cytoplasmatic membrane and nucleous of cells. In addition, the band at 1645 cm^{-1} strongly overlaps the band of −C=C unsaturated groups at 1675 cm^{-1}, depending on chlorophyll pigments.

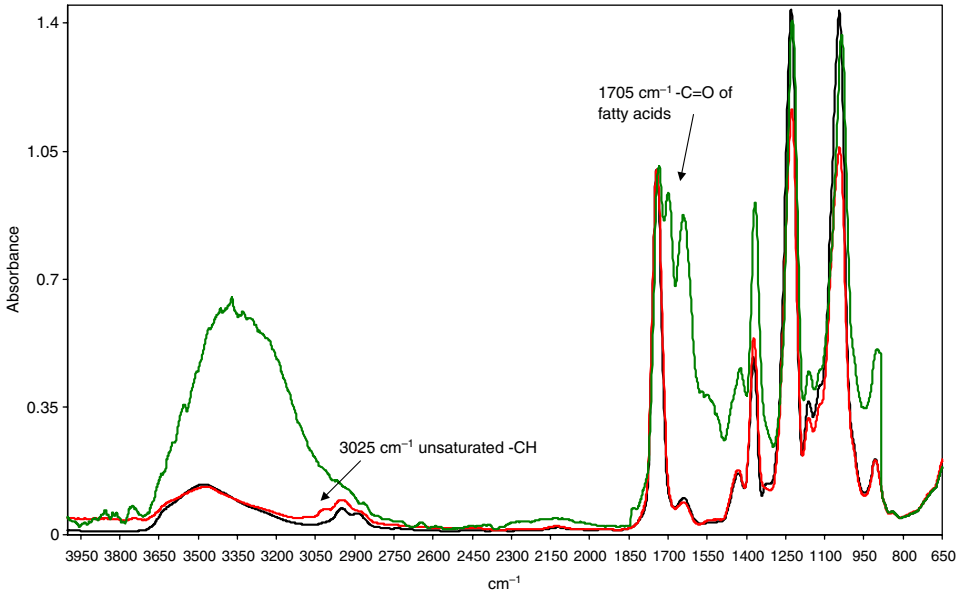

Figure 4. FTIR spectra of control (black) cultures, and cultures exposed to As (red) and As with Triton X-100 (green), showing several molecular modifications. In more detail, the increased bands at 3025 cm^{-1} are related to the increased amount of chlorophyll *a*, whereas the increased band at 1705 cm^{-1} is related to the increased amount of fatty acids (colour online).

These bands are not easily observed from ordinary FTIR spectra, but they become more evident in second-derivative FTIR spectra, not reported here for the sake of brevity.

Other significant bands identified by ordinary and second-derivative spectra are those of phospholipids, carbohydrates and nucleotide groups whose specific assignments are listed in Table 2.

3.3.2. *Molecular changes in exposed algal cells detected by FTIR spectra*

Normalisation of the FTIR spectra allows better investigations of the molecular changes present in the extracts of algal cultures, showing both compositional and structural changes related to pollutant exposure [14]. For example, the band at 3450 cm^{-1} of the hydroxyl group of carbohydrates, proteins and lipids shows changes in intensity related to changes in the concentration of biomolecules, and changes in band shape related to structural polar interactions (Figures 3 and 4). The changes in carbohydrate in *D. tertiolecta.* may depend on the formation of arsenoriboses [12] and are related to modified hydrogen-bond interactions within the cell wall and nucleus, where the interaction between carbohydrates and As occurs.

Molecular modifications observed for proteins and nucleic acids (i.e. the bands at 1650, 1540, 1120 1040 and 830 cm^{-1}) may be related to modifications involving the cytoplasm and nucleus [35]. These types of molecular damage are common when highly polarised atoms interact with DNA [36]. In addition, the structural modification related to the hydrogen bond interaction is confirmed by the $-CH_2$ bending vibration band at 670 cm^{-1}, present as a modification of the band shape (Figure 3); this band is present in all types of biomolecules and is evidence of the structural changes involving carbohydrates proteins lipids and nucleic acids caused by pollutant exposure.

Other bands show modifications related to compositional changes in the involved biomolecules. In the case of exposure to As only, the increased intensity of the bands within 2850 and 3020 cm^{-1},

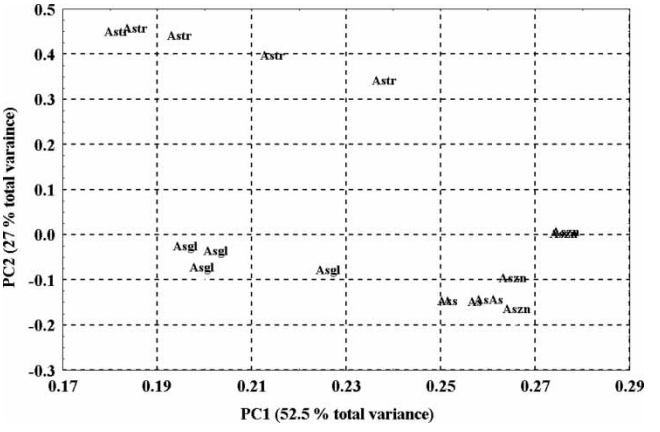

Figure 5. Score plot of PCA applied to FTIR spectra of *Dunaliella tertiolocta* cultures. Astrit, exposure to As and Triton X-100; Asgl, exposure to As and ethylene glycol; Aszn, exposure to As and Zn; As, exposure to As.

belonging to $-CH_2$ groups linked to unsaturated $-C{=}C$ bonds (Figure 4), is partial confirmation of the increased chlorophyll *a* to cell ratio already observed in Vis spectra of *D. tertiolecta* cultures exposed to As (Figure S1, upper left plot – available online only).

Other changes in intensity can be related either to the toxic effect, determining cell number reduction, or to the hormetic effect, stimulating growth and producing an increased cell number with respect to a control sample (Figure 1). In the case of cells exposed to As and Triton X-100, an increased amount of fatty acids (i.e. the band at 1705 cm^{-1}) is observed; this result is analogous to the increased amount of fatty acids reported for *Dunaliella salina* exposed to As [32].

These modified band shapes confirm some similarities observed between toxic and hormetic effect in *D. tertiolecta* cultures under stress conditions [13,14]. However, because of the complexity of the molecular changes shown by Figures 3 and 4, it is hard to compare specific molecular changes and assign them to the type of pollutant exposure by means of a simple visual examination of FTIR spectra. A more complete approach using a multivariate method such as PCA, may allow a better classification of the effects of the examined pollutants on *D. tertiolecta*.

3.3.3. *Classification of molecular modifications by multivariate analysis (PCA) of FTIR spectra*

PCA examination of the FTIR dataset, reported in Figure 5 shows that the first two factors explain 79.5% of the total variance (i.e. included information) present in the spectra and moreover, an evident separation among the different pollutant exposure is detected. Samples exposed to As and As combined with Zn almost overlap and are located in the portion of the score plot characterised by values of the first factor >0.25 and values of the second factor <0.

Samples exposed to As combined with ethylene glycol are characterised by score values of the first factor ranging between 0.19 and 0.23 and score values of the second factor ranging between -0.1 and 0.

Samples exposed to As combined with Triton X-100 are characterised by values of the first factor ranging between 0.17 and 0.25 and values of the second factor ranging between 0 and 0.5. Because of the high similarity observed for cultures having the same type of exposure, it is interesting to point out that molecular modifications observed in presence of the hormetic effect in the mixture of As with Triton X-100 and As with ethylene glycol are comparable with molecular modifications observed in presence of the toxic effect. These results can be explained on the basis

of specific studies reporting how modifications in cell structure arise from an overcompensation in response to alterations in homeostasis, where stimulating and inhibitory pathways take place simultaneously, showing that biostimulated growth is evidence of cell stress [26,37].

At last as far as the score plot is concerned (Figure 5), molecular changes caused by As and As with Zn are highly comparable and yet simultaneously different from other molecular changes otherwise we would not observe the evident separation among groups of samples in the score plot.

The loading values associated with PCA and reported in Figure 6, support the identification of the molecular differences determining the separation among samples observed in Figure 5.

Loadings are the statistical weight (i.e. statistic significance) of the variables (i.e. the wavelengths) [20,21] describing our molecular system, and here they allow the classification

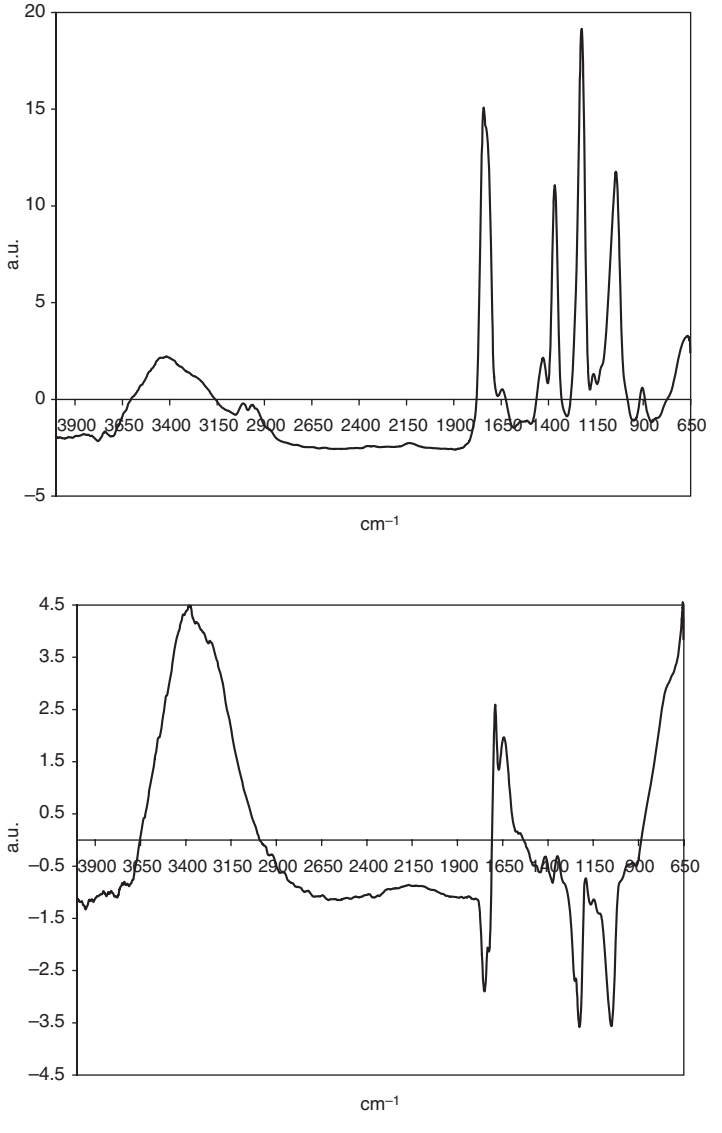

Figure 6. Loading plot of PCA applied to FTIR spectra of *D. tertiolecta* cultures. The first factor is shown in the upper plot and the second in the lower plot. The band intensity shows the statistical significance of the molecular modifications caused by pollutants. The interpretation of these plots is enhanced by the joint examination with Figure 5.

of As-exposed samples. Sample exposed to As and As with Zn have the highest scores in the first factors, as reported previously; as in the first factor, the absorption bands with higher loading values are those at 1745, 1440, 1380, 1240 and 1020 cm^{-1}, this means that according to band assignment (Table 2), the bands of −C=O (ester of fatty acid), $-CH_2$ and −P=O of the phospholipid groups are subjected to the most relevant changes in absorption. Other lower intensity modifications are observed for the bands at 3450 (−OH), 1650 (protein −C=O) and 1160 (carbohydrate C−O−C) cm^{-1}. As far as As with glycol and As with Triton X-100 are concerned, the comparable range of scores in the first factor shows that the above bands are subjected to comparable modifications, which are different from the molecular modifications observed in the As and As with Zn samples.

In terms of the biomolecules involved, this means that the changes in carbohydrates and proteins caused by exposure to As combined with ethylene glycol and Triton X-100 differ from the molecular modifications caused by exposure to As alone and As with Zn, characterised by a pure additive toxic mechanism (Table 1).

This result might be explained by the formation of phytochelatins in the presence of heavy metal pollution and depend on the detoxification mechanism in the alga; in fact, phyochelatin formation has been observed in *D. tertiolecta* exposed to Zn and Cd [38] and in other As-exposed algae such as the green alga *Stichoccus bacillarius* [39] and *Phaeodactilum tricornutum* [40].

The second factors allow further separation of the samples. As with Triton X-100, exposed samples show higher scores in the second factor with respect to the remaining samples. Taking into account the loading values of the second factor, we can see how higher values are encountered for the −OH, −C=O and COOH bands; this confirms that exposure to As combined with Triton X-100 leads to the most relevant changes in fatty acid content with respect to the other types of pollutants already observed (Figure 4).

The different toxic actions of As observed in this study, in both the growth-inhibition tests and the related different molecular modifications in the algal cells agree with a field study on *D. tertiolecta* showing that the total matrix composition of the environmental samples is fundamental to determining toxicity, because the related toxic mechanism may be modified significantly, even in presence of comparable amounts of As [41].

4. Conclusions

FTIR and 3D Vis studies of *D. tertiolecta* cultures show that any type of pollutant mixture that includes As causes specific molecular modifications which are not always comparable with other types of mixtures. These results suggest that research into a molecular biomarker for As exposure by means of *in vitro* tests has to take into account synergic or additive toxic effects, depending on the presence of other pollutants. However, we highlight the proposed FTIR and 3D Vis spectroscopic approaches as fast screening methods to investigate the molecular modifications in probe organisms caused by pollutants because they can be performed on solutions taken from the common growth-inhibition tests. Further developments in the proposed FTIR and 3D Vis spectroscopic methods are their applications for comparing ecotoxicological characteristics of real marine samples.

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