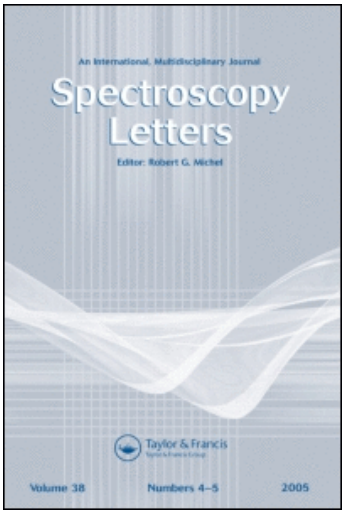

Spectroscopy Letters

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

Effects of Toxic Organotin Compounds on Bacteria Investigated by Micro-Raman Spectroscopy

Ph. Daniel[a]; P. Picart[b]; L. Bendriaa[a]; G. D. Sockalingum[c]; I. Adt[c]; Th. Charrier[d]; M. J. Durand[d]; F. Ergan[e]; M. Manfait[c]; G. Thouand[d]

[a] Laboratoire de Physique de l'État Condensé (LPEC UMR CNRS 6087), Université du Maine, Le Mans, France [b] École Nationale Supérieure d'Ingénieurs du Mans (ENSIM), Université du Maine, Le Mans, France [c] Unité Médian (UMR CNRS 6142), UFR de Pharmacie, Université de Reims (IFR53), Reims, France [d] Université Nantes, UMR CNRS 6144 GEPEA/ERTint 1052 CBAC, IUT GB, Campus de la Courtaisiere, La Roche sur Yon, France [e] Laboratoire d'applications des lipases (LALUM), Université du Maine, IUT de Laval Génie Biologique, Laval, France

To cite this Article Daniel, Ph. , Picart, P. , Bendriaa, L. , Sockalingum, G. D. , Adt, I. , Charrier, Th. , Durand, M. J. , Ergan, F. , Manfait, M. and Thouand, G.(2008) 'Effects of Toxic Organotin Compounds on Bacteria Investigated by Micro-Raman Spectroscopy', Spectroscopy Letters, 41: 1, 19 — 28

To link to this Article: DOI: 10.1080/00387010701799621

URL: http://dx.doi.org/10.1080/00387010701799621

Spectroscopy Letters, 41:19–28, 2008

ISSN: 0038-7010 print/1532-2289 online
DOI: 10.1080/00387010701799621

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

Effects of Toxic Organotin Compounds on Bacteria Investigated by Micro-Raman Spectroscopy

Ph. Daniel,[1] P. Picart,[2] L. Bendriaa,[1] G. D. Sockalingum,[3] I. Adt,[3] Th. Charrier,[4] M. J. Durand,[4] F. Ergan,[5] M. Manfait,[3] and G. Thouand[4]

[1]Laboratoire de Physique de l'État Condensé (LPEC UMR CNRS 6087), Université du Maine, Le Mans, France
[2]École Nationale Supérieure d'Ingénieurs du Mans (ENSIM), Université du Maine, Le Mans, France
[3]Unité Médian (UMR CNRS 6142), UFR de Pharmacie, Université de Reims (IFR53), Reims, France
[4]Université Nantes, UMR CNRS 6144 GEPEA/ERTint 1052 CBAC, IUT GB, Campus de la Courtaisiere, La Roche sur Yon, France
[5]Laboratoire d'applications des lipases (LALUM), Université du Maine, IUT de Laval Génie Biologique, Laval, France

Received 9 September 2006; accepted 28 August 2007.

Address correspondence to Ph. Daniel, Laboratoire de Physique de l'État Condensé (LPEC UMR CNRS 6087), Université du Maine, Le Mans 72085, France. E-mail: philippe.daniel@univ-lemans.fr

ABSTRACT Within the framework of the detection of toxic substances using specific bacteria, the effect of the toxic chemical compound tributyltin chloride (TBT) on the bacterium *Escherichia coli* TBT3 was investigated using a noninvasive approach based on micro-Raman spectroscopy. Various cellular densities with optical density (OD) ranging from 1 to 4 and harvested at different growth phases were induced for 1 h with different TBT concentrations: 1, 3, 5, and 10 μM. As an original contribution, the Raman results show that, in these conditions, the vibrational signatures of bacteria were affected by the presence of the toxic TBT compound, mainly in the signal characteristics from carbohydrates and lipids. Hierarchical cluster analysis (HCA) carried out on the Raman data in these regions makes it possible to classify the spectra in three groups according to the TBT concentration, confirming the progressive influence of the chemical pollutant on the bacterium. This work shows the potentiality for the Raman technique on this subject, even though a low detection threshold corresponding with a concentration of 1 μM of TBT was established.

KEYWORDS *Escherichia coli* TBT3, HCA analysis, organometallic compound, Raman spectroscopy, recombinant bacteria

INTRODUCTION

Tributyltin compounds and derivatives are chemicals that are especially well-known for their numerous applications in the chemical industry. They have been widely used as biocides and also are inserted in industrial paints for their antifouling properties. However, the main problem with such products is their very high toxicity, even at very low concentrations such as nanogram per liter levels.[1] In view of this, since 2001, tributyltin has been included on the European Council list of priority substances in its policy on water,[2] and many research laboratories are now working on the development of chemical analysis methods to detect this type of compound. Alternatively to traditional chemical analysis processes, it appears that recombinant bacteria (RB) could constitute an interesting way to detect chemical compounds because of their high

sensitivity and high-speed data acquisition. RB involves the use of genetically modified bacteria[3] that are able to emit a signal (for instance, weak bioluminescence) when they are in contact with the toxic compound to be identified. Additionally, the intensity of the signal appears to be proportional to the concentration of the toxic compound. In this process, where the toxic chemical "encounters" the bacteria, it is important to understand how the physiologic state of the bacterium changes in presence of tributyltin and how it can be possible to distinguish between the free bacterium and bacterium in contact with the pollutant. To this end, micro-Raman spectroscopy appears as a very convenient analytical method because of its noninvasive and nondestructive character.

Raman spectroscopy is in fact a very convenient method to chemically and structurally investigate all material compounds, of which bacteria now constitute a possible and interesting field of applications. Indeed, the recent technological improvements in Raman spectroscopy, especially in lasers, optics, detectors, and data analysis, make this type of investigation possible. The Raman signal is an inelastic process that occurs when a sample is subjected to a monochromatic laser beam. The observed frequency shifts with respect to that of the laser excitation and corresponds with the vibrational frequencies of the atoms or group of atoms in the studied sample. Note that theoretically the Raman effect is particularly weak, and typically only 1 in 10^8 incident photons are inelastically scattered. Even though Raman spectroscopy does not appear as the most sensitive technique (especially for biological applications), it has the main advantage of being a nondestructive and noninvasive method that will not disturb the studied biological system, which is a major asset. A Raman spectrum is therefore composed of a number of bands that are characteristic of the eigenmodes present in the samples and can be considered as unique for each molecule and compound. Bacteria, like inert materials, are made of different combinations of a number of definite groups of atoms that have characteristic vibrational frequencies. These frequencies are recorded so that the comparison of the Raman spectrum of a material with that of a reference compound gives information about the atoms and bonds existing in the studied material. Note that in biological systems, the main contributions come from proteins, nucleic acids, lipids, glucids, and carbohydrates.

Recent years have seen a huge increase in the use of Raman spectroscopy for biological purposes. As Raman spectroscopy is a whole-cell nondestructive fingerprinting technique, an important advantage is that biochemical information of all cell components is present in the bacterial Raman spectra. Compared with infrared spectroscopy, there is also relatively minor interference with water. The first works that can be found in the literature concerning the applications of Raman spectroscopy in biological systems were reported at the end of the 1980s by Dalterio et al.,[4,5] but the results shown at that period mainly exploit a resonance Raman effect. It has been in the past decade that the Raman technique has reached a high level of sensitivity that enables microorganisms to be identified. Thus, Puppels et al.[6,7] were able to perform a direct and fast identification of microorganisms by applying confocal NIR-Raman spectroscopy or UV Raman technique. Following this, several research groups worked out an identification procedure for bacteria using Raman scattering. The confocal microscopy technique associated with Raman scattering was of great usefulness, making it possible to carry out studies with dispersive systems in the visible range. Then bacteria could be studied by Raman as colonies[8–13] but also according to experimental conditions in low amounts, that is, monolayer or at the single-cell level (see for instance the rather numerous recent works described in Refs. [13–17]). In this respect, only a small number of microorganisms are necessary to obtain reliable spectroscopic data that characterize the cells. Raman scattering has been used to make Gram-type distinction, especially when it is possible to investigate spectral regions that are typical for the outer membrane and cell wall (see for instance the review of Maquelin et al.[14] or the publications of Naumann[18,19]). Additionally, it was recently evidenced that closely related species and subspecies could be distinguished unambiguously using the Raman technique (see for instance the work of Kirschner et al. on the *Enterococcus* group[20] or that of Hutsebaud et al. concerning the species within the *Bacillus subtilis* group[21]), because the spectra reflect their molecular makeup. Raman spectroscopy then offers the possibility of identifying microorganisms at the strain level.[22] As an example in a recent paper, Maquelin et al.[23] evaluate the Raman technique as an epidemiologic tool for *Acinetobacter* strains. One further example shows that the Raman technique

allows the differentiation of *Candida* strains in medical applications more rapidly than by using normal routine analysis.[24] Among more recently published works, special attention has also been paid to the biochemical information that it is possible to extract from the bacterial Raman spectra (as an example, *Cupriavidus mettalidurans*[25]).

From a technical point of view, both the experimental setup and the analysis protocol play an important role in the possibility to obtain information. As previously underlined, the Fourier-transform Raman spectroscopy using a NIR wavelength (generally 1064 nm) has been proved to minimize fluorescent effect (in addition to previously cited papers, see also Refs. [26] and [27]) and was one of the first methods used. Resonance Raman spectroscopy was also selected by researchers because it maximizes the signal together with the absence of parasite luminescence signal, especially in the UV range.[16,28–32] Other enhancement of the signal is also possible using a nonlinear process such as coherent anti-Stokes Raman scattering (CARS; see the review published by Cheng et al.[33]). However, the need for high-power, multiple-wavelength excitation sources has limited the spread of these techniques. A now classic way of enhancing the signal can be found using the surface-enhanced Raman scattering (SERS) method, which makes it possible to improve the signal of a 10^{13} to 10^{15} multiplying factor by using silver colloids or silver surfaces. Many examples of Raman SERS application to bacteria can be found in recent literature,[34–37] but the method of preparation still demands a rather great expertise (except for integrated new systems[38]). A very new and promising approach corresponds with the combination with optical tweezers for the analysis of moving and isolated cells directly in aqueous solution. Optical tweezers allow capture of microorganisms in a liquid medium using a tightly focused near-infrared laser beam, and the same laser generates the Raman effect (see the various papers in Refs. [39–49]). One of the main advantages of this technique is that it allows investigation of the "materials" while naturally suspended in their native environment, without the need for particle adhesion to a surface. Additionally, in the case of biological samples, no concentration is required and the measurements can be done at the single cell level.

Dispersive Raman scattering in the visible range ($\lambda = 785$ nm or 647 nm) also appears to be very often used because of the recent technological improvements in detectors and, as previously indicated, because of the coupling with confocal microscopy, which makes it possible to obtain a very good spatial resolution. Even though it can appear as a more "classic" method, the Raman dispersive technique in the visible range is still probably one of the simplest among Raman possibilities and is now easier to use especially for nonspecialists. Within the framework of potential applications, for instance in the field of biosensors, this method was then selected here.

In summary, Raman spectroscopy now appears as a very powerful tool to study bacterial and biological systems (see some applications cited in Refs. [50–53]), however, as far as we know, the study of interaction between a chemical compound and bacteria has not been investigated yet, in spite of the numerous potential applications. In this framework, this paper is then devoted to the effect of toxic organotin compounds on bacteria by micro-Raman spectroscopy. We have studied the effect of the organotin compounds such as tributyltin on the *Escherichia coli* TBT3 bacterial strain by micro-Raman spectroscopy. Note that tributyltin chloride is a compound of chemical formula $(C_4H_9)_3ClSn$ and will be labeled TBT in this paper. There is also the dichloride form DBT $(C_4H_9)_2Cl_2Sn$. The strain *Escherichia coli* TBT3 was isolated after a random insertion of the *lux*AB (promoter-less) genes of *Vibrio harveyi* into the *Escherichia coli* DH1 chromosome. Durand et al. (2003)[54] demonstrated that the Ec::*lux*AB TBT3 strain is specifically induced by TBT and DBT (with Cl, Br, or I as the halogen group) with the central tin atom required for light production. The detection limits were found to be 0.08 μM for TBT ($26\ \mu g\ L^{-1}$) and 0.0001 μM for DBT ($0.03\ \mu g\ L^{-1}$) with a linear range of 1 logarithm.

This paper is divided into two main parts: the first part concerns the experimental procedures used, and the second discusses the main results obtained by micro-Raman spectroscopy.

MATERIALS AND METHODS

Preparation of Bacteria

Bacterial Strains

One of the main difficulties in investigating bacteria by Raman scattering concerns the preparation of the "biological material" for such experiments, and it therefore appears essential to set up a precise experimental

procedure, compatible with all restrictions imposed by these "living" cells. In our case, the bacterium TBT3 is an *Escherichia coli* strain that was obtained after random insertion of the *lux*AB genes from *Vibrio harveyi* into *E. coli* DH1.[55,56] A colony of the bacterium TBT3 was cultivated in 20 mL glucose medium with 20 μL tetracycline (concentration 1%) at 37°C under continuous stirring (250 rpm) for about 14 h (overnight culture). The use of tetracycline makes it possible to keep the plasmid pFUSLUX in the strain during the growth.[55] Under these experimental conditions, the culture had a final optical density of about 1, providing a sufficient biomass for the spectral analysis. For our Raman experiments, four concentrations of tributyltin were selected: 1 μM, 3 μM, 5 μM, and 10 μM. Organotin stock solutions (600 μM) were prepared with 60 mL ethanol 70% adjusted to 100 mL with seawater.

The induction of the bacterium TBT3 with TBT is carried out by addition of 2.5 mL TBT stock solution with 5 mL cell suspension. Cells were induced for 1 h without shaking at 30°C before analysis by Raman spectroscopy. In a previously published work, Horry et al.[57] have shown that the effect of TBT on the bacterium TBT3 is clearly evidenced by a bioluminescence effect (with addition of decanal to develop the luminescence emission) of which the intensity reaches a maximum after an induction time of 1-h. This observation leads us to think that this 1 h duration is a critical time corresponding with a maximized biochemical effect of the pollutant on the bacterium and then was selected for our experiments in order to be evidenced by Raman scattering.

Cell suspensions were centrifuged twice at 4°C (5 min, 4000 × *g*) to harvest the cells. Note that because the cells are in the same growth phase, we can assume that the cells will give the same spectroscopic signal with and without TBT. Before loading samples on the microscope, 5 μL of each suspension was put down on a zinc selenide carrier (ZnSe) and dried for 15 min. This substrate (ZnSe) is suitable because it does not possess any Raman signature in the studied spectral range (600–1750 cm^{-1}). This drying process is now a well-admitted routine method as previously described in many papers found in the literature (see for instance Refs. [11, 16, 20, 21, 23, 25, and 26] where the process is fully described as being close to ours). In spite of its current use, it cannot be excluded that dehydration induces some modification of the physiologic state of the bacteria, however this study (as the others published elsewhere concerning Raman scattering in bacteria) can bring valuable comparative information.

Growth Medium

Glucose medium[11] with a C/N/P ratio (w/w/w) of 100/10/1 was prepared with 1 L tap water filtered through an 0.45-μm membrane filter: D-(+)-glucose monohydrate (1.376 g), NH_4Cl (0.1919 g), K_2HPO_4 (0.028 g), NaCl (5 g), yeast extract (0.5 g), and tryptone (1 g). The pH was adjusted to 7. After sterilization of the medium by autoclaving 20 min at 120°C, 100 μL of a sterile oligoelement solution (SL7) was added. SL7 medium was prepared as follows for 1 L distilled water: HCl (13 mL), $ZnSO_4 \cdot 7H_2O$ (1.44 g), $MnCl_2 \cdot 4H_2O$ (1 g), H_3BO_3 (620 mg), $CoCl_2 \cdot 6H_2O$ (1.9 g), $CuCl_2 \cdot 2H_2O$ (170 mg), $NiCl_2 \cdot 6H_2O$ (240 mg), $Na_2MO_4 \cdot 2H_2O$ (360 mg). Note that this medium is the best for the induction of the strain by TBT as previously determined by Durand et al.[51]

Micro-Raman Spectroscopy

Raman spectra were recorded on a commercial multichannel Raman micro-spectrometer (Lab Ram; Horiba Jobin Yvon Raman Division, Lille, France) and the experimental setup is described in Ref. [58]. The 785-nm radiation from an Ar^+-pumped Ti-Sa laser (Spectra Physics, Les Ulis, France) was used for excitation in combination with a 100× long-working-distance objective (NA = 0.9) of an Olympus BX41 transmission and reflection illumination microscope (Olympus, Rungis, France) for focus and collection of Raman scattered light. The confocal hole was set to 800 μm. The dispersive system is composed of a single grating with 950 groves/mm, and the Raman signal is collected by a front illuminated CCD detector cooled by thermoelectric Peltier air-effect (produced by Horiba Jobin-Yvon; 1024 × 256 chip size). A notch filter rejects the Rayleigh contribution. Figure 1 displays the experimental set-up used here.

A nominal laser light power was adjusted to a maximum value of 50 mW. However, due to the optical configuration of the spectrometer, the real power focused on the biological sample does not exceed 5 mW. The thermal exchange is favored by the ZnSe substrate, and no degradation of the samples was optically observed for each experiment. The laser

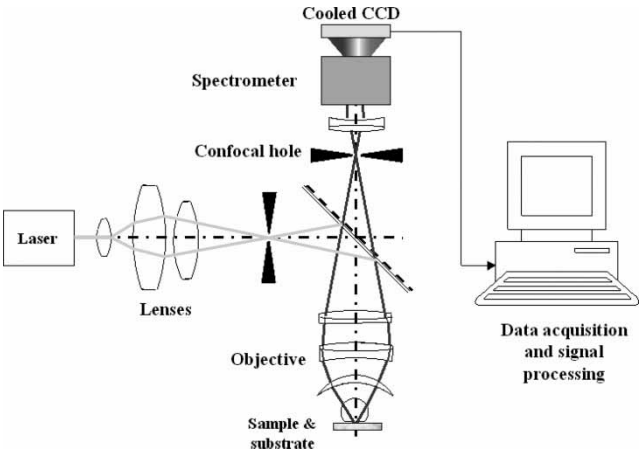

FIGURE 1 **Schematic Layout Showing the Experimental Setup for Micro-Raman Measurements.**

beam focused on the TBT3 bacterial biomass typically gives a spot size of 1–2 μm. As an example, a typical picture of the biomass of TBT3 bacteria under the microscope is illustrated in Fig. 2. The laser beam was targeted on the biomass visually, using a TV monitor and a motorized XY stage. The Raman signal was optimized by adjusting the laser focus with a real-time readout; the spectrum was then acquired in the useful spectral range 600–1750 cm^{-1} with an integration time of 200 s. Five types of samples were analyzed by Raman spectroscopy: the bacterium TBT3 without induction by TBT and the bacterium TBT3 induced with 1 μM, 3 μM, 5 μM, and 10 μM of TBT, respectively. All data acquisition and control of experimental parameters were carried out using LabSpec 4.03 (Horiba Jobin Yvon).

Data Analysis

For each sample, three series of independent measurements were carried out (corresponding with three independent bacterial cultures), and for each set, the Raman experiment was repeated six times to check the reproducibility of the measurement and to minimize the possible artifacts and parasitic signals. For further analysis, it was considered at each time the average of the six measured spectra. Each considered average spectrum was preprocessed with a 9-point Savitsky–Golay function corresponding with a denoising routine (smoothing filter), background corrected with a polynomial function, and vector normalized using the Labspec software.

However, because the bacteria systems are complex on a biochemical level for a spectroscopic analysis, a classic "peak to peak" Raman analysis with assignment of each line is not usually possible. In these bacterial systems, as very often observed and published elsewhere, the Raman spectra look highly similar (see Refs. [21, 23, 25, and 27]) and only mathematical data processing allows the extraction of information. Then, in order to exploit the spectra, a multivariate statistical method can be used (Ref. [9] fully depicts these mathematical procedures applied to bacteria) such as principal components analysis (PCA), discriminant function analysis (DFA), or hierarchical cluster analysis (HCA). The selected method here was HCA because it makes it possible to obtain information on the dissimilarity between a data set or "individuals," which are here the Raman spectra with neighboring characteristics. The data analysis was done following Ward's clustering algorithm and the Euclidian distance measure to generate a dendrogram (Software OPUS 3.1 NT from Bruker Optics, Ettlingen, Germany, dedicated to spectroscopy applications). To summarize, the results displayed as dendrograms quantify the degrees of heterogeneity corresponding with a quantification of the Euclidian distance between data. The HCA analysis was tested on four different spectral regions and on the full spectrum.

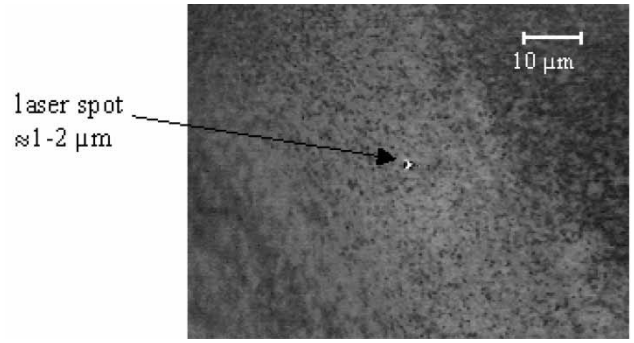

FIGURE 2 **Typical Bacterial Biomass Imaging Under 100× Long-Work-Distance Objective of the Raman Microscope.**

RESULTS AND DISCUSSION

As has already been stated, this work is devoted to testing the potential of micro-Raman spectroscopy to detect differences in the physiologic state of bacteria according to their environment, namely in this case, in the presence of the toxic compound TBT. Figure 3 exhibits typical micro-Raman spectra of the bacteria *E. coli* TBT3 with and without the presence of the toxic compound tributyltin. Based on literature data reported for other microorganisms,[8–38] the main

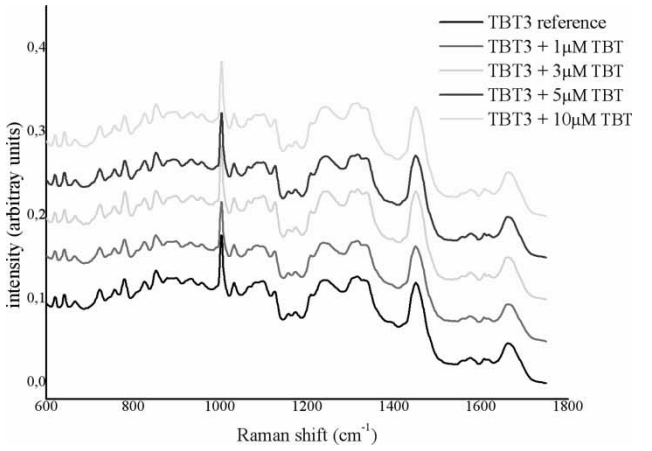

FIGURE 3 **Micro-Raman Spectra of *E. coli* TBT3 Bacteria Induced with TBT and Un-Induced.**

components contributing to the Raman spectra, especially the characteristic bands specific to nucleic acids, lipids, proteins, and carbohydrates, can be identified. Table 1 summarizes these bands and their assignment.

As shown by the spectra and in Table 1, Raman spectroscopy provides a chemical "fingerprint" of the studied "material." According to the data obtained here, the spectral profiles appear visually very similar between the tributyltin-induced and uninduced bacteria. However, even if the Raman spectrum reflects the chemical composition, the variations in the evolution of the physiologic state of the bacterium in contact with the toxin can hardly be detected; the Raman spectra appear very similar. These similar profiles can be explained by the fact that very small variations can be masked by the many common structural and functional components contained in microorganisms. It can also be considered that the effect of the toxin is negligible and thus undetected by a classic analysis of Raman spectra, mainly because of the complexity of these biological systems. As previously underlined, it is therefore necessary to apply other methods that are able to differentiate between closely related data. In order to compare our data, in this study we have used HCA analysis, which allows us to classify the spectra according to degree of heterogeneity between them. One interest of this method is to perform the analysis either on the full frequency range spectrum or on restricted frequency regions in order to surround the possible differences between Raman spectra. As a first step, the spectra were analyzed by considering the full spectral range 600–1750 cm^{-1} where all characteristic vibrational components of a bacterium are present. In this calculation, it seems very difficult to differentiate between TBT-induced and -uninduced bacteria, indicating that the differences are probably masked if we consider the full frequency range (even if the effect of TBT on bacteria is effective). Then, four restricted frequency domains were tested, and finally it appears that the only zone where the chemical compound TBT affects the bacterium TBT3 corresponds with the spectral range 900–1200 cm^{-1}. This region is associated with the carbohydrates and lipids in the bacteria. These results are summarized in Figs. 4a–d representing the dendrograms calculated on the considered frequency zone 900–1200 cm^{-1}.

According to these dendrograms, especially those exhibited in Figs. 4a and 4b, the Raman spectra from the bacterium *E. coli* TBT3 alone can be easily distinguished from that of the strain in contact with the toxic TBT. This is partially true for toxin concentrations of 3 μM and 5 μM. Additionally, an easy distinction between the concentrations 3 μM and 10 μM is possible. This means that corresponding spectra are distinguishable between the weak and strong concentrations and they each form an independent class.

However, in Fig. 4d, for the weakest concentrations (1 μM and 3 μM), there is no clear-cut distinction between reference spectra (i.e., uninduced TBT3) and TBT3 induced with the toxin TBT. Furthermore, according to the results obtained for a bacterial suspension with optical density of 1, it appears very difficult with the Raman scattering to detect the effect of TBT at a concentration less than 1 μM. Thus, a threshold seems to be reached by using Raman spectroscopy to characterize the chemical evolution. It can be considered that such an amount of pollutant is not

TABLE 1 **Bands Observed in Micro-Raman Spectra of TBT3 Bacteria and Assignment to Main Biomolecular Components**

Assignment	Raman shift (cm^{-1})
Nucleic acids	782
Phenylalanine	1001
Phenylalanine	1031
Carbohydrates	1097
Lipids	1129
Amide III	1240–1310
Nucleic acids	1336
Lipids	1449
Lipids	1480
Amide II	1571
Amide I	1664

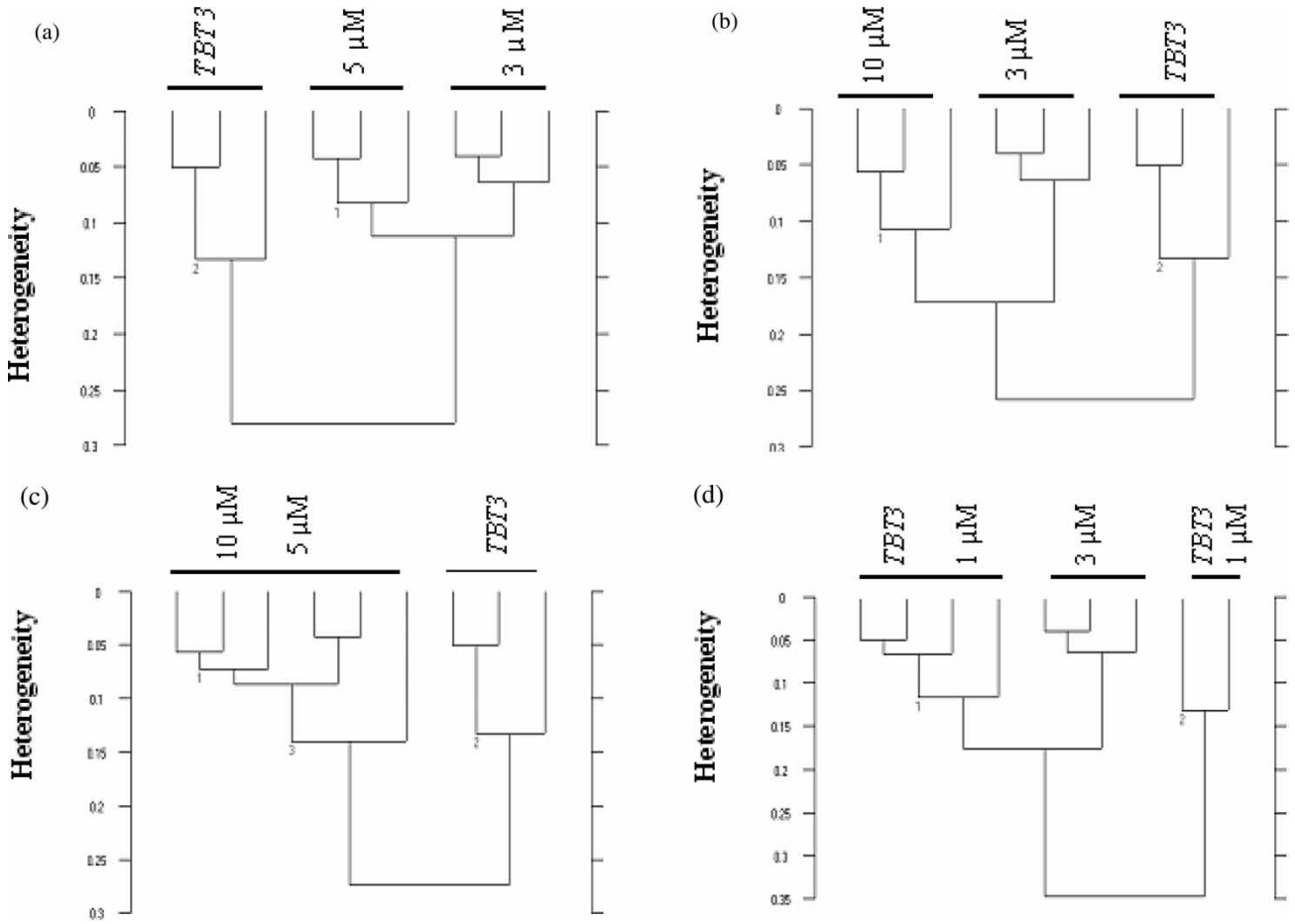

FIGURE 4 **Dendrograms Resulting from Hierarchical Cluster Analysis (HCA): (a) Three Families (TBT3, 3 µM and 5 µM TBT); (b) Three Families (TBT3, 3 µM and 10 µM TBT); (c) Three Families (TBT3, 5 µM and 10 µM TBT); (d) Three Families (TBT3, 3 µM and 1 µM TBT).**

enough to induce a structural modification of the cell. For concentrations of 5 µM and 10 µM, an overlapping of the spectra is also observed. One possible interpretation is connected to saturation effect on the bacteria. Actually, the stress of the bacterium submitted to an important amount of tributyltin induces a maximum effect on certain functional groups present in the system.

To summarize this study, three classes can be distinguished. Each class contains bacteria that have the same physiologic state:

- Class 1: It is possible to separate spectra of bacteria according to concentrations of TBT to which they are exposed. A distinction is then possible between reference, 3 µM, and 5 µM concentrations but also between reference, 3 µM, and 10 µM.
- Class 2: There is no effect of the tributyltin on the TBT3 bacterium. This zone concerns the weak concentrations in TBT, lower than 1 µM. In this case, the physiologic state of bacteria does not change in contact with the TBT, according to the analysis of the Raman data.
- Class 3: This class concerns the strong concentrations, higher than 5 µM. It seems that there is an effect of saturation of the bacteria toward the toxin, explaining the lack of discrimination.

CONCLUSIONS

In this work, the effect of tributyltin on the *E. coli* TBT3 bacteria was monitored by micro-Raman spectroscopy, which is known to be sensitive to the chemical composition and environment of the studied sample. We show that, after evaluating the data with HCA, tributyltin had an effect on carbohydrate and lipid contents, which correspond with the spectral range 900–1200 cm^{-1} of the Raman spectra. According to the clustering obtained after the HCA analysis, it can be noted that the physiologic state of

the bacteria changes with the concentration of TBT to which they are exposed. In addition, changes were observed for TBT concentrations higher than 1 μM. At this early stage of interpretation, we can hypothesize that TBT could interact with the cell wall of the bacteria. Indeed, *Escherichia coli* is a Gram-negative bacterium and consequently contains lipopolysaccharide (LPS) embedded in the external membrane. The latter forms a complex lipid–protein structure. In contact with TBT, both lipid and polysaccharide contained in the LPS can be modified, leading to a modification of the Raman spectra. Actually, Radecka et al.[59] and Zielinska et al.[60] demonstrated that trialkyltin and dialkyltin interacted with an artificial bilayer lipidic membrane that led to a local depolarization.

This study shows the potential of micro-Raman spectroscopy as a nondestructive and noninvasive spectroscopic technique for monitoring the interaction of bacteria with exogenous molecules. Raman spectroscopy coupled with multivariate analytical methods therefore represents a valuable investigative method to study the effect of an environmental pollutant on bacteria, even though at this stage of the investigation, it is not possible to determine an unknown TBT concentration with only the Raman information obtained from bacteria. However, it can be considered that if the vibrational signal can be enhanced, the Raman technique could then be directly used as a very quick analytical method for detection of pollutants in biological media. In conclusion, this work appears as a first promising step of a more extensive study, especially considering the recent developments observed in Raman spectroscopy (such as confocal laser tweezer Raman spectroscopy), which will be very useful to expand the potentiality of this subject.

ACKNOWLEDGMENTS

This research was supported by the CER 2000–2006 grant, research program no. 18035 (Ville de la Roche sur Yon, Conseil Général de Vendée, Conseil Régional des Pays de la Loire, Ministère Français chargé de la Recherche). We are indebted to all colleagues of the Laboratoire d'Applications des Lipases (LALUM) for all suggestions, especially for advice in biology. The authors are grateful to Prof. Alison Bates, English native and English teacher at Université du Maine (Le Mans, France), for her help in language proof reading and improvement.

REFERENCES

1. Fent, K. Ecotoxicology of organotin compounds. *Crit. Rev. Toxicol.* **1996,** *2*, 1–117.
2. Decision No. 2455/2001/EC of the European Parliament and of the Council of 20 November establishing the list of priority substances in the field of water policy and amending Directive 2000/62/EC. *Official Journal of European Communties* L331 44, December 15, 2001.
3. Bendriaa, L.; Picart, P.; Daniel, Ph.; Horry, H.; Durand, M. J.; Thouand, G. Versatile device for on-line and in-situ measurement of growth and light production of bioluminescent cells. *Sensors Actuators B* **2004,** *103* (1–2), 115–121.
4. Dalterio, R. A.; Nelson, W. H.; Britt, D.; Sperry, J.; Purcell, F. J. A resonance Raman microprobe study of chromobacteria in water. *Appl. Spectrosc.* **1986,** *40* (2), 271–272.
5. Dalterio, R. A.; Baek, M.; Nelson, W. H.; Britt, D.; Sperry, J.; Purcell, F. J. The resonance Raman microprobe detection of single bacterial-cells from a chromobacterial mixture. *Appl. Spectrosc.* **1987,** *41* (2), 241–244.
6. Puppels, G. J.; De Mul, F. F. M.; Otto, C.; Robert-Nicoud, M.; Arndt-Jovin, J.; Jovin, J. Studying single living cells and chromosomes by confocal Raman microspectroscopy. *Nature* **1990,** *347*, 301–303.
7. Puppels, G. J.; Colier, W.; Olminkhof, J. H. F.; Otto, C.; De Mul, F. F. M.; Greve, J. Description and performance of a highly sensitive confocal microspectrometer. *J. Raman Spectrosc.* **1991,** *22* (4), 217–225.
8. Schrader, B.; Dippel, B.; Fendel, S.; Keller, S.; Löchte, T.; Riedl, M.; Schulte, R.; Tatsch, E. NIR FT Raman spectroscopy—a new tool in medical diagnostics. *J. Mol. Struct.* **1997,** *408–409*, 23–31.
9. Goodacre, R.; Timmins, E. M.; Burton, R.; Kaderbhai, N.; Woodward, A. M.; Kell, D. B.; Rooney, P. J. Rapid identification of urinary tract infection bacteria using hyperspectral whole organism fingerprinting artificial neural networks. *Microbiology* **1998,** *144*, 1157–1170.
10. Schrader, B.; Dippel, B.; Erb, I.; Keller, S.; Löchte, T.; Schulz, H.; Tatsch, E.; Wessel, S. NIR Raman spectroscopy in medicine and biology: results and aspects. *J. Mol. Struct.* **1999,** *480–481*, 21–32.
11. Maquelin, K.; Choo-Smith, L.-P.; van Vreeswijk, T.; Endtz, H. Ph.; Smith, B.; Bennett, R.; Bruining, H. A.; Puppels, G. J. Raman spectroscopic method for identification of clinically relevant microorganisms growing on solid culture medium. *Anal. Chem.* **2000,** *72* (1), 12–19.
12. Maquelin, K.; Choo-Smith, L.-P.; Endtz, H. Ph.; Bruining, G. J.; Puppels, G. J. Raman spectroscopic studies on bacteria. *Proc. SPIE* **2000,** *4161*, 144–150.
13. Schuster, K. C.; Urlaub, E.; Gapes, J. R. Single-cell analysis of bacteria by Raman microscopy: spectral information on the chemical composition of cells and on the heterogeneity in a culture. *J. Microbiol. Methods* **2000,** *42* (1), 29–38.
14. Maquelin, K.; Kirschner, C.; Choo-Smith, L.-P.; van den Braak, N.; Endtz, H. Ph.; Smith, B.; Naumann, D.; Puppels, G. J. Identification of medically relevant microorganisms by vibrational spectroscopy. *J. Microbiol. Methods* **2002,** *51* (3), 255–271.
15. Rösch, P.; Schmitt, M.; Kiefer, W.; Popp, J. The identification of microorganisms by micro-Raman spectroscopy. *J. Mol. Struct.* **2003,** *661–662*, 363–369.

16. López-Diéz, E. C.; Goodacre, R. Characterization of microorganisms using UV resonance Raman spectroscopy and chemometrics. *Anal. Chem.* **2004,** *76* (3), 585–591.
17. Huang, W. E.; Griffiths, R. I.; Thompson, I. P.; Bailey, M. J.; Whiteley, A. S. Raman microscopic analysis of single microbial cells. *Anal. Chem.* **2004,** *76* (15), 4452–4458.
18. Naumann, D. Infrared and NIR Raman spectroscopy in medical microbiology. *Proc. SPIE* **1998,** *3257*, 245–257.
19. Naumann, D. Infrared and Raman spectroscopy of biological materials. In *Practical Spectroscopy Series*; Gremlich, H. U., Yan, B., Eds.; Marcel Dekker: New-York, 2000; pp. 323–377.
20. Kirschner, C.; Maquelin, K.; Pina, P.; Ngo Thi, N. A.; Choo-Smith, L. P.; Sockalingum, G. D.; Sandt, C.; Ami, D.; Orsini, F.; Doglia, S. M.; Allouch, P.; Mainfait, M.; Puppels, G. J.; Naumann, D. Classification and identification of Enterococci: a comparative phenotypic, genotypic, and vibrational spectroscopic study. *J. Clin. Microbiol.* **2001,** *39* (5), 1763–1770.
21. Hutsebaut, D.; Vandroemme, J.; Heyrman, J.; Dawyndt, P.; Vandenabeele, P.; Moens, L.; de Vos, P. Raman microspectroscopy as an identification tool within the phylogenetically homogeneous 'Bacillus subtilis'–group. *Syst. Appl. Microbiol.* **2006,** *29*, 650–660.
22. Yang, H.; Irudayaraj, J. Rapid detection of foodborne microorganisms on food surface using Fourier transform Raman spectroscopy. *J. Mol. Struct.* **2003,** *646* (1), 35–43.
23. Maquelin, K.; Dijkshoorn, L.; van der Reijden, T. J. K.; Puppels, G. J. Rapid epidemiological analysis of Acinetobacter strains by Raman spectroscopy. *J. Microbiol. Methods* **2006,** *64* (1), 126–131.
24. Ibelings, M. S.; Maquelin, K.; Endtz, H. P.; Bruining, H. A.; Puppels, G. J. Rapid identification of *Candida* spp. in peritonitis patients by Raman spectroscopy. *Clin. Microbiol. Infection* **2005,** *11* (5), 353–358.
25. De Gelder, J.; De Gussem, K.; Vandenabeele, P.; de Vos, P.; Moens, L. Methods for extracting biochemical information from bacterial Raman spectra: an explorative study on Cupriavidus metallidurans. *Anal. Chim. Acta* **2007,** *585* (2), 234–240.
26. Huang, W. E.; Griffiths, R. I.; Thompson, I. P.; Bailey, M. J.; Whiteley, A. S. Raman microscopic analysis of single microbial cells. *Anal. Chem.* **2004,** *76* (15), 4452–4458.
27. Choo-Smith, L. P.; Maquelin, K.; van Vreeswijk, T.; Bruining, H. A.; Puppels, G. J.; Ngo Thi, N. A.; Kirschner, C.; Naumann, D.; Ami, D.; Villa, A. M.; Orsini, F.; Doglia, S. M.; Lamfarraj, H.; Sockalingum, G. D.; Manfait, M.; Allouch, P.; Endtz, H. P. Investigating microbial (micro)colony heterogeneity by vibrational spectroscopy. *Appl. Environ. Microbiol.* **2001,** *67* (4), 1461–1469.
28. Manoharan, R.; Ghiamati, E.; Dalterio, R. A.; Britton, K. A.; Nelson, W. H.; Sperry, J. F. UV resonance Raman spectra of bacteria, bacterial spores, protoplasts and calcium dipicolinate. *J. Microbiol. Methods* **1990,** *11* (1), 1–15.
29. Ghiamati, E.; Manoharan, R.; Nelson, W. H.; Sperry, J. F. UV Resonance raman spectra of Bacillus spores. *Appl. Spectrosc.* **1992,** *46* (2), 357–364.
30. Nelson, W. H.; Manoharan, R.; Sperry, J. F. UV Resonance Raman studies of bacteria. *Appl. Spectrosc. Rev.* **1992,** *27* (1), 67–124.
31. Wu, Q.; Hamilton, Th.; Nelson, W. H.; Elliot, S.; Sperry, J. F.; Wu, M. UV Raman spectral intensities of *E. coli* and other bacteria excited at 228.9, 244.0, and 248.2 nm. *Anal. Chem.* **2001,** *73*, 3432–3440.
32. Jarvis, R. M.; Goodacre, R. Ultra-violet resonance Raman spectroscopy for the rapid discrimination of urinary tract infection bacteria. *FEMS Microbiol. Lett.* **2004,** *232* (2), 127–132.
33. Cheng, J. X.; Xie, X. S. Coherent anti-Stokes Raman scattering microscopy: instrumentation, theory, and applications. *J. Phys. Chem. B* **2004,** *108* (3), 827–840.
34. Sockalingum, G. D.; Lamfarraj, H.; Beljebbar, A.; Pina, P.; Delavenne, M.; Witthuhun, F.; Allouch, P.; Manfait, M. Vibrational spectroscopy as a probe to rapidly detect, identify, and characterize micro-organisms. *SPIE Proc. Biomed. Appl. Raman Spectrosc.* **1999,** *3608*, 185–194.
35. Zeiri, L.; Bronk, B. V.; Shabtai, Y.; Czégé, J.; Efrima, S. Silver metal induced surface enhanced Raman of bacteria. *Colloids Surfaces Physicochem. Eng. Aspects* **2002,** *208* (1–3), 357–362.
36. Jarvis, R. M.; Goodacre, R. Discrimination of bacteria using surface-enhanced Raman spectroscopy. *Anal. Chem.* **2004,** *76* (1), 40–47.
37. Jarvis, R. M.; Brooker, A.; Goodacre, R. Surface-enhanced Raman spectroscopy for bacterial discrimination utilizing a scanning electron microscope with a Raman spectroscopy interface. *Anal. Chem.* **2004,** *76* (17), 5198–5202.
38. Yan, F.; Vo-Dinh, T. Surface-enhanced Raman scattering detection of chemical and biological agents using a portable Raman integrated tunable sensor. *Sensors Actuators B* **2007,** *121* (1), 61–66.
39. Xie, C. G.; Dinno, M. A.; Li, Y. Q. Near-infrared Raman spectroscopy of single optically trapped biological cells. *Optics Lett.* **2002,** *27*, 249–251.
40. Xie, C. G.; Li, Y. Q. Confocal micro-Raman spectroscopy of single biological cells using optical trapping and shifted excitation difference techniques. *J. Appl. Phys.* **2003,** *93* (5), 2982–2986.
41. Xie, C. G.; Li, Y. Q.; Tang, W. Study of dynamical process of heat denaturation in optically trapped single microorganisms by near-infrared Raman spectroscopy. *J. Appl. Phys.* **2003,** *94* (9), 6138–6142.
42. Chan, J. W.; Motton, D.; Rutledge, J. C.; Keim, N. L.; Huser, T. Raman spectroscopic analysis of biochemical changes in individual triglyceride-rich lipoproteins in the pre- and postprandial state. *Anal. Chem.* **2005,** *77* (18), 5870–5878.
43. Creely, C. M.; Singh, G. P.; Petrov, D. Dual wavelength optical tweezers for confocal Raman spectroscopy. *Optics Commun.* **2005,** *245* (1–6), 465–470.
44. Creely, C. M.; Volpe, G.; Singh, G. P.; Soler, M.; Petrov, D. Raman imaging of floating cells. *Optics Express* **2005,** *13* (16), 6105–6110.
45. Singh, G. P.; Volpe, G.; Creely, C. M.; Grotsh, H.; Geli, I. M.; Petrov, D. The lag phase and G1 phase of a single yeast cell monitored by Raman microspectroscopy. *J. Raman Spectrosc.* **2006,** *37* (8), 858–864.
46. Chan, J. W.; Esposito, A. P.; Talley, C. E.; Hollars, C. W.; Lane, S. M.; Huser, T. Reagentless identification of single bacterial spores in aqueous solution by confocal laser tweezers Raman spectroscopy. *Anal. Chem.* **2004,** *76*, 599–603.
47. Xie, C. G.; Mace, J.; Dinno, M. A.; Li, Y. Q.; Tang, W.; Newton, R. J.; Gemperline, P. J. Identification of single bacterial cells in aqueous solution using confocal laser tweezers Raman spectroscopy. *Anal. Chem.* **2005,** *77*, 4390–4397.

48. Taylor, D. S.; Chan, J. W.; Zwerdling, T.; Lane, S. M.; Tuscano, J.; Huser, T. Laser tweezers Raman spectroscopy detects differences between normal human lymphocytes, activated lymphocytes and leukaemia. *Blood (ASH Annual Meeting Abstracts)* **2005,** *106* (11), 4530.
49. Taylor, D. S.; Chan, J. W.; Zwerdling, T.; Lane, S. M.; Ihara, K.; Huser, T. Laser tweezers Raman spectroscopy detects individual neoplastic and normal hemotopoietic cells. *Blood (ASH Annual Meeting Abstracts)* **2004,** *106* (11), 4531.
50. Notingher, I.; Hench, L. L. Raman microspectroscopy: a non invasive tool for studies of individual living cells in vitro. *Expert Rev. Med. Devices* **2006,** *3* (2), 215–234.
51. Naumann, D. FT-infrared and FT-Raman spectroscopy in biomedical research. *Appl. Spectrosc. Rev.* **2001,** *36* (2–3), 239–298.
52. Petrich, W. Mid-infrared and Raman spectroscopy for medical diagnostics. *Appl. Spectrosc. Rev.* **2001,** *36* (2–3), 181–237.
53. Petry, R.; Schmitt, M.; Popp, J. Raman spectroscopy—a prospective tool in the life sciences. *Chem. Phys. Chem.* **2003,** *4* (1), 14–30.
54. Durand, M. J.; Thouand, G.; Dancheva-Ivanova, T.; Vachon, P.; DuBow, M. S. Specific detection of organotin compounds with a recombinant luminescent bacteria. *Chemosphere* **2003,** *52* (1), 103–111.
55. Guzzo, A.; DuBow, M. S. Construction of stable, single-copy luciferase gene fusions in *Escherichia coli*. *Arch. Microbiol.* **1991,** *156*, 444–448.
56. Briscoe, S. F.; Diorio, C.; DuBow, M. S. Luminescent biosensors for the detection of tributyltin and dimethyl sulfoxide and the elucidation of their mechanism of toxicity. In Environmental biotechnology: principles and applications. Moo–young, M., Anderson, W. A., Chakrabarty, A. M., Eds.; Kluwer Academic Press: Dordrecht, Boston, London, **1996**; 645–655.
57. Horry, H.; Durand, M. J.; Picart, P.; Bendriaa, L.; Daniel, Ph.; Thouand, G. Development of a biosensor for the detection of organotin compounds. *Environ. Toxicol.* **2004,** *19*, 342–345.
58. Piot, O.; Autran, J. C.; Manfait, M. Assesment of cereal quality by micro-Raman analysis of the grain molecular composition. *Appl. Spectrosc.* **2002,** *56* (9), 1132–1138.
59. Radecka, H.; Zielinska, D.; Radecki, J. Interaction of organic derivatives of tin (IV) and lead (IV) with model lipid membranes. *Sci. Total Environ.* **1999,** *234* (1–3), 147–153.
60. Zielinska, D.; Radecka, H.; Radecki, J. Contribution of membrane surface charge in the interaction of lead and tin derivatives with model lipid membranes. *Chemosphere* **2000,** *40* (3), 327–330.