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A Mechanistic Approach to Tributyltin (TBT) Sorption by Marine Microflagellated Alga *Pavlova lutheri*

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The sorption of tributyltin (TBT) by phytoplankton cells was studied by exposing the marine microflagellated alga *Pavlova lutheri* grown in batch culture to TBT chloride concentrations ranging from 0.37 to 74 nM (0.1 to 21 $\mu g\, l^{-1}$) for a 24-hour period. The phytotoxicity of TBT was indicated by a decrease in cell density at all TBT concentrations used. TBT was tightly bound to the surface cell and was only washed out by a strong acidic solution (pH $\leqslant$ 3) while inner cell-absorbed TBT was only recovered by means of an organic solvent. The sorption of TBT by *P. lutheri* occurred via two mechanisms: a passive ionic surface adsorption followed by a facilitated intracellular absorption. The coupling/uncoupling of these two mechanisms was dependent upon the contamination level used. The cell surface adsorption of TBT was best described by the Freundlich adsorption model whereas the obstruction of the facilitated absorption mechanism was apparently related to the toxic activity of TBT towards cell components.

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INTRODUCTION

Freshwater[1–3] and marine[4–8] microalgae can accumulate high levels of tributyltin (TBT), an organometallic biocide widely used in aquatic environments since the 1970s.[9–11] TBT is an amphiphilic compound and shows characteristics of both metal ions and hydrophobic materials with respect to its partitioning between dissolved and particulate phases. The uptake of TBT by phytoplankton cells has usually been considered as a simple partitioning mechanism driven mostly by its hydrophobic character[1, 6] and most studies on phytoplankton species have reported TBT accumulation as total burdens[1, 2, 6] without discriminating between surficial adsorbed and inner absorbed fractions. This lack of discrimination between fractions resulted in a large discrepancy in published bioconcentration factors[1, 2] (BCFs), which were not related to chemical and biological characteristics of living cell membranes. As a consequence, measured BCF values from experimental exposures or from cells collected in contaminated areas (BCF ranging from 3000 to 30 000) were always much higher than those calculated from hydrophobic partitioning coefficients[12] (BCF $\approx$ 300) based on hydrophobic indices, such as the octanol–water coefficient (K_{ow}) and the organic carbon content of exposed cells. This discrepancy can be tentatively attributed to strong interactions of TBT cations with anionic sites located on the cell surface, somewhat similar to those observed at the surface of mineral particles.[13, 14] As phyto-

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plankton cells exhibit large surface/volume ratios, an extensive surficial adsorption without an intracellular absorption would contribute to an anomalously high BCF.[15]

The binding of TBT onto cells could result from ionic interactions between the positively charged tin atom and anionic sites on the cell surface such as sulphate and carboxylate groups,[16, 17] but it could also result from electrostatic interactions of micellar TBT to the cell membrane or direct binding to proteins and glycoproteins of this membrane.[18] Once a TBT molecule has interacted with the cell surface it could be immobilized at the surface or it could diffuse into the cells. Absorption could occur either by diffusion through the lipidic layer[19] or by a facilitated interaction with a membrane carrier via an ion membrane channel.[20, 21] Surficial interactions of TBT are not yet clear but the whole process can be seen as a reaction between a dissolved TBT molecule and ligands present at the cell surface.[22, 23]

The main objective of this study was to study the sorption mechanism of TBT on a marine phytoplankton species, *Pavlova lutheri*, by evaluating the influence of various aqueous concentrations on its partitioning process between seawater and the phytoplankton cells, and by investigating its distribution between the cellular surface and cellular cytoplasm.

MATERIALS AND METHODS

Growth conditions

Axenic cultures of the prymnesiophycea *Pavlova lutheri* (obtained from the INRS—Oceanologie collection) were grown in medium F/2[24] in 4-litre flasks at 17 °C, with magnetic stirring (the rate was kept below 60 rpm to avoid resuspension of settled cells), and a 14 h:10 h light/dark photocycle at a light intensity of 140 $\mu Em^{-2} s^{-1}$. The sterile medium was inoculated to approximately 2×10^4 cell ml^{-1} or lower when needed. The density of algae in each culture was estimated from a 2-ml sample withdrawn without stopping the magnetic stirrer. Cells were counted on a Neubauer haemacytometer (for each reported value, $n = 4$–6 counts and CV = 15%).

TBT accumulation by algae

The influence of the aqueous concentration of TBT on its distribution between the extracellular surface and the inner compartment of cells was investigated by exposing populations of *P. lutheri* for 24 h to different concentrations of TBTCl ranging from 0.37 nM to 74 nM (0.1 to 21 μg/l). For each experiment, 100 μl of a solution of TBTCl (Aldrich Co., USA) in ethanol at the appropriate concentration was added to each culture when algae were in their exponential growth phase. Control experiments consisted of cultures to which 100 μl of pure ethanol were added. Also, to study the surface complexation process, two different populations (in cell density and biomass) of *P. lutheri* were contaminated at an identical concentration of TBTCl (37 nM), also for 24 h.

Phase separation

The distinction between the surface ionic sorption and the inner accumulation of TBT was operationally defined by a separation protocol described in detail elsewhere.[4] Briefly, cells were centrifuged and the pellet was resuspended in filtered (0.45 μm) seawater acidified to pH 3 with HNO_3 to desorb TBT from the surface of the cells without disrupting the plasma membrane (see below). The suspension was then centrifuged, the supernatant was filtered, and the TBT found in the resulting solution was considered as the acid-extractable TBT fraction or surface-adsorbed TBT (TBT_{ads}). TBT organically extracted from the pellet and the filter represented the organic-extracted TBT fraction or absorbed TBT (TBT_{abs}).

Influence of pH on the recovery of TBT from algae

To assess the effect of the pH of the seawater washing solution on the TBT recovery, a 4-litre culture of *P. lutheri* was exposed to 37 nM TBTCl for 24 h. The culture was then divided into three subsamples and each subsample was extracted using seawater with the pH adjusted at 5, 3 and 1, respectively. Microscopic examination of cells after extraction revealed that *P. lutheri* cells were very resistant to acidic treatment, and lysis of some cells (10%) occurred only for those washed with seawater acidified at pH 1. Seawater washing solution at pH 3 was used for all other analyses to avoid any lysis and the results on adsorption were extrapolated for an extraction at pH 1 by multiplying the value obtained at pH 3 by a factor of 2.5 (calculated from the results shown in Table 1 below).

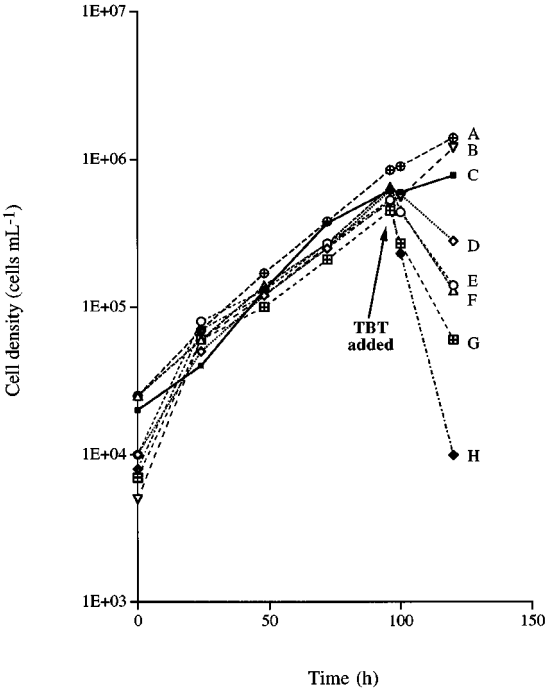

Figure 1 Variation of cell density in *Pavlova lutheri* batch cultures exposed to various concentrations of tributyltin chloride for 24 h. A and B are controls; C = 0.37; D = 3.7; E = 11.1; F = 18.5; G = 37.0; H = 74.0 nM TBTCl.

Organotin analysis

Organotins extracted from various aqueous phases and from lyophilized phytoplankton were converted to hydrides following a method described elsewhere[4] and derivatized organotins were analysed and quantified by GC–MS[4] and GC–FPD.[25] The detection limit was 1 pmol of TBTH injected (CV = 12%).

RESULTS AND DISCUSSION

Effects of TBT on cell density

The most obvious effect of increasing TBT concentration on cell growth was a rapid reduction of the cell density after 24 h (Fig. 1). A concentration as low as 0.37 nM TBT induced a reduction of the growth rate, confirming Beaumont and Newman's[7] results obtained with the same species. This result is in contrast with a previous study[4] where a concentration of 18.5 nM TBTCl did not seem to adversely affect the grow rate of *P. lutheri* exposed to TBT in a flow-through culture system. The low tolerance of *P. lutheri* to TBT poisoning when grown in a batch culture system is attributed to the different nutritional responses and physiological state of cells cultured by different techniques.[26] It could also be related to the accumulation of toxic exudate in the batch culture.

pH dependence of TBT recovery

The quantity of TBT extracted from the cellular surface increased from 8.5 nmol g^{-1} to 307.6 nmol g^{-1} as the pH of the washing solution was lowered from 5 to 1 (Table 1). The increasing desorption of TBT from the cellular surface as a result of increasing proton concentration is a clear indication that the binding mode of TBT onto cells is related to ionic interactions between cationic TBT and some anionic sites at the cell surface. At low pH, protons compete for biological sorption sites and succeed in dislodging TBT cations. These results are consistent with previous work[16] on pH behaviour of metal sorption onto marine microalgae (*Cladophora serica, C. rupestries, Enteromorpha linza, E. intestinales, Rhizoclonium* and *Polysiphonia* spp.) concluding that metals were exchanged at carboxylate sites. Similarly, an acid–base equilibrium can be written for TBT species at the cell surface (Eqn [1]):

$$RCOO^{-}\ {}^{+}SnBu_3 + H_3O^{+} \rightleftarrows Bu_2SnOH_2^{+} + RCOOH \qquad [1]$$

NMR analysis of TBT species in solution[27] confirmed the predominance of $Bu_3SnOH_2^+$ and Bu_3SnCl at pH < 7, whereas carbonato species were detected at seawater pH approx. 8.

The concentration of intracellular TBT (Table 1) extracted with hexane after acidic washing was almost constant (mean = 55.4 ± 2.6 nmol g^{-1}), suggesting cells were not damaged and inner TBT was not lost even at pH 1. Table 1 also provides a mass-balance calculation for total TBT recovery in each extraction. As expected, the maximum recovery (87%) was obtained only for the extraction with seawater at pH 1. Missing TBT in the extraction (13% of the nominal concentration) would represent losses from adsorption onto the vessel walls, and possibly from a partial degradation of TBT to dibutyltin (DBT) during the exposure period.[4] TBT not recovered by an aqueous wash at pH 5 or 3 was not available to the organic extraction, confirming the ionic character of its binding into

Table 1 Effects of pH of aqueous extractions on TBT recovery from *P. lutheri* cells exposed to 37 nM TBTCl for 24 h in batch culture

Aqueous extraction pH	Acid-extractable TBT (nmol g^{-1})	Organic-extractable TBT (nmol g^{-1})	Total TBT in algal fraction (nM)	TBT dissolved in water (nM)	Total TBT recovered (nM)	(%)
5	8.5	58.4	1.3	24.9	26.2	71
3	118.9	52.1	3.6	24.9	28.5	77
1	307.6	55.7	7.1	24.9	32.0	87

the cellular wall.[23] Results in Table 1 support the hypothesis that the uptake of TBT by *P. lutheri* alga can be discriminated into two fractions operationally defined as the surface cellular TBT (TBT_{ads}), a fraction extracted in aqueous acidic solutions, and the intracellular TBT (TBT_{abs}), a lipidic fraction extracted only by an organic solvent which can disrupt cells and reach the cytoplasm content. Such a fractional separation was only possible with the marine alga *P. lutheri* exhibiting a remarkable resistance to lysis in acidic solutions.

Surface adsorption versus intracellular absorption

The comparison of surface $[TBT]_{ads}$ (Fig. 2) with inner $[TBT]_{abs}$ (Fig. 3) as a function of increasing TBT concentrations in solution, $[TBT]_w$, revealed quite different patterns of accumulation for both compartments. For a given contamination level in the culture, the concentration of $[TBT]_{ads}$ was always higher than the concentration of $[TBT]_{abs}$ and this difference increased as TBT concentrations increased. A similar trend was previously observed during the first days of exposure of *P. lutheri* to TBT in a chemostat[4] and seems to be a common behaviour for *P. lutheri.*

The variation of $[TBT]_{abs}$ as a function of the contamination level followed a nonlinear relationship where the incorporation increased with water contamination level to a maximum of ≈ 50 nmol g^{-1} at 18.5 nM TBT in water, but dropped to about 34 nmol g^{-1} and levelled off for the highest concentrations. The pattern of Fig. 3 is quite similar to those obtained in cellular membrane transport studies where ion transport through membrane channels decreases at higher substrate levels as a result of a saturation process.[28] This behaviour is also well known in enzyme kinetics and is usually attributed to an excess substrate inhibition. These similarities suggest that the mechanism responsible for the incorporation of TBT into cells might be blocked at high TBT concentrations by the progressive inhibition of ion movements in saturated membrane channels. In support of this hypothesis, the inhibition of the mitochondrial inner membrane anion channel by some triorganotin chlorides has been reported and TBTCl was identified as the most potent inhibitor of this membrane channel system.[20] Similar studies have not yet been conducted for unicellular algae, but our results suggest that the internal transport of TBT in *P. lutheri* might be, at least in part, a facilitated process rather than a passive

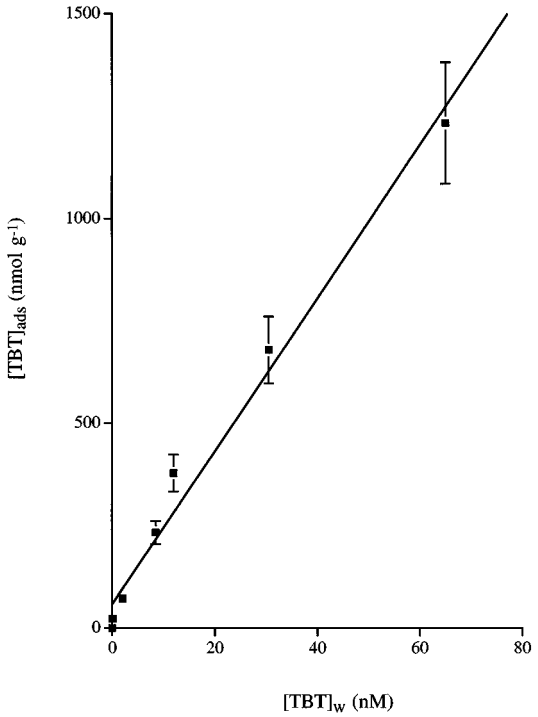

Figure 2 Relationship between surficial adsorption of TBT by *Pavlova lutheri* after 24 h exposure and TBTCl concentrations in cultures. Error bar = 1 SD ($n = 3$).

diffusion[29] where the membrane is modified during the absorption process.

Surface adsorption isotherm

The surface sorption of TBT on the cell wall (Fig. 2) offers some similarities to the reported sorption of organotins on clay minerals[13, 14, 30, 31] which could be described by the classical Langmuir (Eqn [2]) and/or Freundlich (Eqn [3]) isotherms:

$$C_{ss}C_{eq} = C_{ss}/C_m + 1/C_mL \quad [2]$$

$$C_{eq} = KC_{ss}^{1/n} \quad [3]$$

where C_{ss} is the solute concentration in the solution at the steady state; C_{eq}, the amount of solute adsorbed on the solid at equilibrium; C_m, the maximum adsorption of the sorbent; L, a constant related to the adsorption energy; K, the Freundlich adsorption capacity parameter; and $1/n$ the Freundlich intensity parameter. Adsorption data from Fig. 2 were better described by a Freundlich isotherm ($r^2 = 0.966$) rather than a Langmuir adsorption isotherm ($r^2 = 0.610$) and the linearized expression of the former gives Eqn [4]:

$$\log[TBT]_{ads} = \log K + 1/n \log[TBT]_w \quad [4]$$

where $[TBT]_{ads}$ is given in nmol g^{-1}; $[TBT]_w$ is the aqueous concentration of TBT in nM at steady-state after 24 h of exposure. The Freundlich parameters ($\log K = 1.87$ and $1/n = 0.63$) obtained in this case were comparable with those reported for the adsorption of TBT onto silty clay sediment.[13] This calculation assumes that missing TBT (13%) was not adsorbed onto cells. However, even if all the missing TBT was actually adsorbed and not acid-extracted, a calculation with 100% recovery shows that the effect on the isotherms ($\log K$ increased to 2.02) does not change the intensity parameter ($1/n$) since the slope is not changed.

The adsorption of TBT is clearly a surface-related phenomenon, as postulated by the isotherm model, with various adsorption energies and possible interactions among adsorbed species.[30] Equations [4] holds only for a given phytoplankton species, assuming that the adsorption surface ($m^2\ g^{-1}$) is constant for a given mass of sorbent. However, this condition could change with phytoplankton culture conditions as the cell number (and thus the total adsorption surface can vary) for a given biomass, depending on the cell size and the physiological state of the algal population.

To approach the adsorption models, it appeared more appropriate to consider the adsorption of TBT on an individual cell basis (mol $cell^{-1}$) by Equation [5]:

$$C_s = TBT_{ads}/N_{cell} \quad [5]$$

where C_s is the amount of TBT at the surface of a single cell, TBT_{ads} is the quantity of TBT adsorbed on cells in a given volume of sample and N_{cell} is the maximum number of cells in the same volume. The maximum number of cells for the period of exposure has been used because dead cells are not eliminated in a batch culture system without consumers and thus are still acting as non-living sorbent particles. The results of Fig. 2 can be re-assessed as the variation of surface TBT per cell C_s, as a function of the total TBT per cell C_{max} corresponding to the quantity of TBT a cell would accumulate if all the TBT in solution were available to the cells (initial amount of TBT/N_{cell}). Again, the results (Fig. 4) are best fitted by linearized form of the Freundlich isotherm (Eqn [6]):

$$\log C_s = \log K + 1/n \log C_{max} \qquad r^2 = 0.974 \quad [6]$$

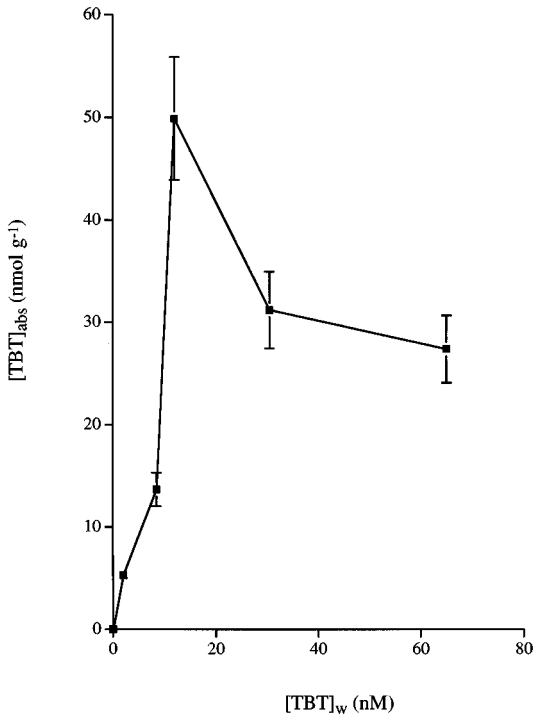

Figure 3 Changes of inner absorption of TBT by *Pavlova lutheri* after 24 h exposure for different TBTCl concentrations in cultures. Error bar = 1 SD ($n = 3$).

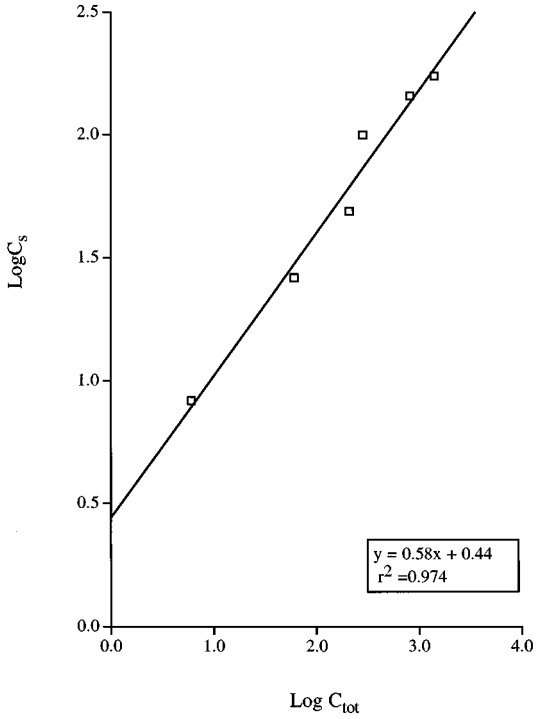

Figure 4 Relationship between surficial adsorption of TBT per cell (log C_s) and total uptake of TBT per cell (log C_{tot}).

where log $K = 0.44$ and $1/n = 0.58 \pm 0.14$ ($P = 0.05$). This Freundlich model is applicable to the surface sorption of TBT on *P. lutheri* for the whole range of concentrations we used, and seems to be a good predicting tool.

The adsorption of some dissolved metals onto algal suspensions (*Chlamydomonas rheinhardii*) has been studied previously[32] and the results were interpreted in terms of surface complex formation equilibria using adsorption isotherms. When comparing TBT results with those obtained for dissolved metals, it becomes evident that the behaviour of TBT and metallic cations towards the cellular surface are quite similar, being described by similar approaches.

A possible saturation process

Results from experiments where the cell density of the algal population was increased as the contamination level was kept constant are reported in Table 2. C_{max} values provide the maximum quantity of TBT a cell would accumulate if all the TBT in solution were available to sorption. C_s and C_i are experimental values calculated from $[TBT]_{ads}$ and $[TBT]_{abs}$, cellular density and biomass. For all three experiments, total TBT measured at the surface and in individual cells ($C_s + C_i$) was always much lower than C_{max}, suggesting the presence of a mechanism affecting the uptake process. When expressing the ratio between ($C_s + C_i$) and C_{max} as the uptake efficiency (%), it becomes clear that the efficiency of the sorption increased as the cellular density increased. Under an undisturbed dilution process, an increasing number of cells should take up fewer TBT molecules, but the efficiency should not change. The improved efficiency of cells as TBT concentrations decrease might be attributed to a saturation process taking place at low cellular density, as the number of available TBT cations is much greater than the number of available biological sites at the cell surface. Following the conceptual model of metal–organism interactions proposed by Campbell,[23] a trace metal cation approaching and entering the cellular wall of a microorganism will encounter a complex layer of macromolecules before reaching the plasma membrane. A saturation of this matrix of negatively charged sites through which the metal must migrate might reduce the rates of association and dissociation of the metal cation with and from negative sites, and then reduce its progression inside the matrix. We observed that TBT is very tightly bound to the surface of the cell, which means that TBT can find 'secure sites' inside the cell wall (also called the cortex), sites that even small protons can reach only with difficulty. In other words, too many TBT cations led to a low uptake efficiency because receptor sites at the cell wall (not at the plasma membrane) were saturated.

Intracellular absorption process

The intracellular absorption of TBT is a much more complex process and simple linearized equations cannot be applied. Introducing values of $[TBT]_{abs}$ from Fig. 3 into Eqn [5], the amount of intracellular TBT per single cell (C_i) can be calculated and compared with surface-adsorbed TBT. The progressive inhibition of the incorporation of TBT with increasing adsorption is highlighted when C_i is plotted against surface TBT per cell C_s (Fig. 5). The intracellular content reached a maximum of 13×10^{-19} mol cell^{-1} and decreased to less than 4×10^{-19} mol cell^{-1} at the highest value of surface TBT per cell (173×10^{-19} mol cell^{-1}), representing a C_s/C_i

Table 2 TBT accumulation (10^{-19} mol cell^{-1}) as a function of the cellular density in compartments of *P. lutheri* cells exposed to 37 nM TBT for 24 h

Expt no.	Cellular density (10^5 cells ml^{-1})	Biomass (wet wt) (mg l^{-1})	C_{max}	C_s	C_i	C_s+C_i	Uptake efficiency (%)
I	4.5	9.8	822	137	6	143	17
II	7.8	15.4	474	96	26	122	26
III	18.2	40.3	203	68	13	81	40

ratio of 43. It should be mentioned that the decrease in C_i with the increase in C_s is not an artifact due to the presence in the batch culture of dead cells having no active incorporation of TBT. Previous results in a chemostat[4] also indicated a decrease of TBT_{abs} with increasing level of contamination of the culture medium under a constant renewal of the population of algae when no dead cells were present.

The relationship between C_i and C_s is clearly not controlled by a simple dose/response process and might again be a saturation mechanism, this time at the plasma membrane, related to the decrease in the membrane fluidity. As TBT is lipid-soluble and possibly penetrates the cell by a direct interaction with membrane lipids,[33] an excess of TBT crossing the membrane might affect its fluidity by modifying the arrangement and interactions of the different lipids and proteins composing the membrane. The effect of a decrease in membrane fluidity is a reduction of the mobility of carrier molecules and even a complete obstruction of their movement.[33] Furthermore, TBT has been identified as a specific inhibitor of photophosphorylation and $NADP^+$ reduction.[34] When isolated chloroplasts from *Euglena gracilis* were exposed to TBT, phosphorylation was inhibited at tremendously low concentrations ranging from 5 to 10 nM, e.g. TBT levels even lower than those used in the present experiment (Fig. 2). Khan[34] also observed that a very low toxicant/target ratio (1 mol of TBT to 120 mol chlorophyll) gave complete inhibition of photophosphorylation, indicating the very specific binding between TBT and phosphorylation sites. The partial inhibition of photophosphorylation in *P. lutheri* was probably the toxic mechanism which resulted in a marked decrease in cell density at a TBT concentration as low as 3.7 nM (Fig. 1). It also means a possible inhibition of any facilitated transport of TBT through cellular membranes, since a facilitated transport would require energy and only phosphorylation could produce the required ATP. Triphenyltin chloride is also known to inhibit ATP formation and coupled electron transport in isolated chloroplasts.[35]

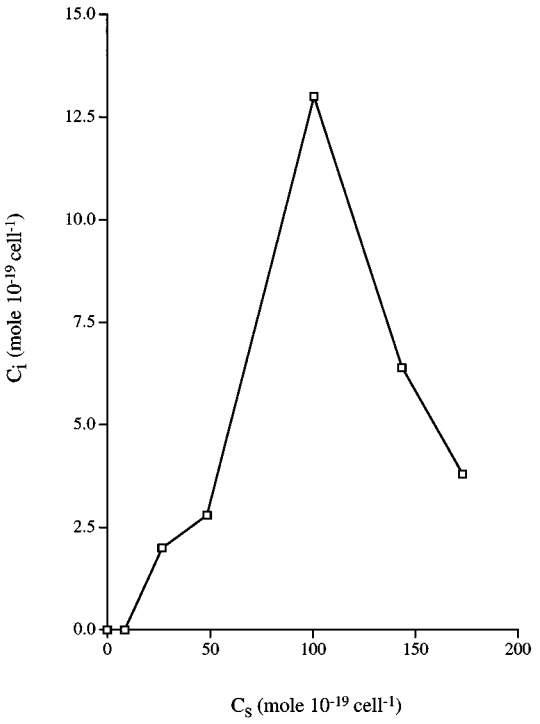

Figure 5 Charges of the inner absorption of TBT per cell (C_i) as a function of the surficial adsorption per cell (C_s).

In summary, our mechanistic approach provides answers about the dual process of TBT sorption onto *P. lutheri*:

(1) The accumulation of TBT by *Pavlova lutheri* occurred via two mechanisms acting in tandem, an ionic surface adsorption and a facilitated intracellular absorption. The coupling of these mechanisms depends on the contamination level to which algae are exposed and is sensitive to a saturation process acting at the

cellular surface.

(2) The surficial adsorption of TBT appears to be mainly an ionic process which is best described by the Freundlich adsorption model. TBT cations are very tightly bound to the cell wall due to their amphiphilic properties.

(3) The toxic effects of TBT on photophosphorylation in chloroplasts might be the explanation for the low quantity of TBT incorporated in the cell at high concentrations of TBT in solution and at the surface of the cell.

These findings also illustrate the relative complexity of what seems to be a simple uptake of an organometal cation by phytoplankton cells. The amphiphilic character of TBT introduces an additional parameter, usually not present with dissolved trace metals, to be considered when attempting to understand its chemical interactions with both cell surfaces and plasmal membranes. Further work in progress, conducted at very low concentrations (e.g. < 100 ng l^{-1}) to avoid a direct toxic effect of TBT on chloroplasts, should allow the determination of uptake kinetics and provide more information on specific binding of organotins with biological constituents of the cell wall, an ill-defined protective layer of most unicellular phytoplankton.

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