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ARTÍCULO

The ignored stowaways: worldwide dispersion of exotic microalgae species through the biofouling recovering the ships underwater body

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ABSTRACT

Invasion by introduced species cause huge environmental damage and economic (estimated in $138 billion in USA). Marine ecosystems are specially affected by introduced species of toxin-producing microalgae. Ships ballast water has been considered the major vector in dispersion of phytoplankton. However, most ships do not use ballast water. Alternatively, we propose that the biofouling recovering the underwater body of ships is the main cause of microalgal dispersion. Antifouling paints (containing tributyltin, TBT or other toxics) are used to coat the bottoms of ships to prevent biofouling. After sampling biofouling recovering the underwater body of ships we demonstrate that numerous coastal, oceanic and toxin-producing microalgae species proliferates attached on bottoms of ships directly on TBT antifouling paint. These microalgae species should be resistant variants because antifouling paints rapidly destroy sensitive wild type microalgae. Consequently, the key to explain microalgae

species transport via ships biofouling is know the mechanisms that allow to these species to survive long time attached to antifouling paint. A fluctuation analysis demonstrate that genetic adaptation by rare spontaneous mutation, which occurs by chance prior to antifouling exposure is the mechanism allowing adaptation of microalgae to antifoulig paints and their dispersion in the ships biofouling. Around 3 TBT-resistant mutants per each 10^{-4} wild type sensitive cells occurs in microalgal population. This assures a rapid colonization of ships bottoms to travel long-distances.

Key words: Adaptation; Biofouling; HABs; Harmful algae; Introduced species; TBT.

RESUMEN

Los polizones ignorados: dispersión por el mundo de las especies de microalgas exóticas mediante el «biofouling» que recubre el fondo de los barcos

La introducción de especies invasoras puede causar grandes problemas medioambientales y económicos (estimados en 138 billones de $ en USA). Los ecosistemas marinos se ven especialmente afectados por la introducción de microalgas tóxicas. El agua de lastre de los barcos está considerada como el mayor vector de dispersión de fitoplancton. Sin embargo, la mayoría de los barcos no tienen lastre de agua. Como alternativa, proponemos que el biofouling que recubre los barcos es la principal causa de dispersión de microalgas. Se utilizan pinturas antifouling (conteniendo tributil-estaño, TBT u otros tóxicos) para recubrir la obra viva de los barcos previniendo el biofouling. Despues de diversos muestreos de la obra viva en barcos demostramos que numerosas especies de microalgas costeras, oceánicas y productoras de toxinas son capaces de proliferar adheridas a la obra viva de los barcos, directamente sobre la pintura TBT antifouling. Estas microalgas deben ser variantes resistentes porque el TBT rápidamente destruye las microalgas sensibles. Consecuentemente, la clave para explicar el transporte de las especies de microalgas en el biofouling de los barcos es conocer los mecanismos que permiten a las especies sobrevivir mucho tiempo sobre la pintura

antifouling. Un análisis de fluctuación ha demostrado que la adaptación genética debida a raras mutaciones espóntaneas, que ocurren anteriormente a la exposición al TBT, es el mecanismo que le permite a las microalgas adaptarse a la pintura antifouling y su posterior dispersión en el biofouling. Hay alrededor de tres mutantes resistentes al TBT por cada 10^{-4} células sensibles en la población. Esto asegura la rápida colonización de la obra viva de los barcos para viajar largas distancias.

Palabras clave: Adaptación; Biofouling; HABs; Algas tóxicas; Especies introducidas; TBT.

1. INTRODUCTION

Numerous introduced species (also called exotic, non-indigenous, or alien species) proliferates worldwide outside its native distributional range usually as a consequence of human activities. Invasion by introduced species can change the functions of ecosystems causing unpredictable emergent novelties, environmental damage and loss in biodiversity (1). Economic cost assigned to introduced species in USA was estimated in $138 billion (2).

Marine ecosystems are specially affected by introduced species (3). As an example, the introduced algae *Caulerpa taxifolia* represents a major risk for sublittoral Mediterranean ecosystems (4, 5). Invasion of toxin producing phytoplankton is particularly worrisome because of their potentially devastating impacts on aquaculture, fishery and public health (6-8). Ballast water has been considered the major vector by which (mainly phytoplankton) have invaded ecosystems worldwide where they did not previously occur (9-14). Consequently, biocide treatments and other management strategies are carried out in ballast water to minimize introduction of alien species (15, 16).

The hypothesis of ballast water as mechanisms of dispersion of marine organisms is attractive. However, few ships (mainly tankers and containers) used ballast water (17). Most ships (including recreational yachts) do not use ballast water. In contrast, any boat or ship afloat has an underwater body that can be rapidly colonized by thousands of marine species of algae, crustacean and mollusc attaching themselves to the hull forming a dense biofouling.

We propose that the biofouling recovering the underwater body of ships is a source to disperse algal species, which contributes to bio-invasion by alien species at least as efficiently as ballast water.

However, antifouling paints are widely used to coat the bottoms of ships to prevent biofouling. During the 1960s the chemical industry developed efficacious anti-fouling paints using metallic compounds in particular the organotin compounds tributyltin (TBT) and triphenyltin (TPT) (reviewed in 18). TBT and TPT are very toxic for algae, crustacean and molluscs. These organotin compounds have been extensively used in ships worldwide. Although some countries recently banned TBT and TPT (19, 20), the alternative antifouling paints are also based on other toxic compounds such as cupper salts (20).

In this work we demonstrate that: i) numerous species of microalgae travel great distances forming the biofouling recovering the underwater body of ships, ii) these microalgal species are resistant to anti-fouling paints, and iii) the antifouling paint-resistant microalgae arose by rare spontaneous mutation.

2. MATERIALS AND METHODS

2.1. Sampling of biofouling recovering the underwater body of ships

Microbiological hyssops were scraped against the TBT-antifouling paint of boats that arriving to Sagunto Port, Valencia, Spain after large oceanic journeys (i.e. From Africa, America). The hyssops were immersed in culture flaks (Greiner, Bio-One Inc., Longwood NJ, USA) with 20 ml of BG-11 medium (Sigma Aldrich Chemie, Taufkirchen, Germany), and stored at 15 ºC in darkness until laboratory identification of microalgae species. Microalgae were identified in fresh samples using settling chambers under and inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany).

2.2. Experimental organism

Laboratory experiments were performed with the unicellular *Chlorophyta* (strain *Dc1M*), from the Algal Culture Collection of the Universidad Complutense (Madrid). The strain was grown axenically in cell culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of Sigma algal culture medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), at 20º C under continuous light of 60 mol photom m^{-2} s^{-1} over the waveband 400-700 nm. It was maintained in mid-long exponential growth by serials transfers of a cell inoculum to fresh medium (once every month). Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures. Cultures were maintained as axenic as possible and only cultures without detectable bacteria were used in the experiments.

2.3. Toxicity test: effect of TBT on growth rate and photosynthesis performance

A stock solution of TBT (Sigma) was prepared in BG-11 medium to obtain serial dilutions of 0, 1, 3, 10, and 30 ppm. Each experimental culture was inoculated with 6×10^6 cells from mid-log exponentially growing cultures. Two replicates of each concentration of TBT, as well as two unexposed controls, were prepared.

The toxic effect of the TBT was estimated by calculating acclimated maximal growth rate (m) in mid-log exponentially growing cells, derived from the equation:

$$N_t = N_0 e^{mt} \quad (21)$$

where t = 7 days, the time that cultures were exposed to different dose of TBT, and N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively. Experiments and controls were counted blind (i.e., the person counting the test did not know the identity of the tested sample), using a Uriglass settling chamber (Biosiga, Cona, Italy) and an inverted microscope (Axiovert 35, Zeiss).

The effective quantum yield (Φ PSII) was also measured in triplicates of experiments and controls of both species using a ToxY-

PAM fluorimeter (Walz, Effeltrich, Germany) at seven different time points (0.1, 1, 10, 12, 24, 48 and 72 h). Effective quantum yield was calculated as follows:

$$\Phi \text{ PSII} = (F'_m - F_t) / F'_m \quad (22)$$

F'_m, F_t, maximum and steady-state fluorescence of light-adapted cells, respectively.

2.4. Fluctuation analysis of TBT-sensitive → TBT-resistant transformation

A modified Luria-Delbruck fluctuation analysis (23) was performed to distinguish resistant cells that had their origin in random spontaneous pre-selective mutations (mainly prior to TBT exposure) from those arising through acquired post-selective adaptation (during the exposure to TBT) (Figure 1). Two different sets of experimental cultures were prepared. In the set 1 experiment, 96 culture flasks were inoculated with $N_0 = 10^2$ cells (a number small enough to reasonably ensure the absence of pre-existing mutants in the strain). Cultures were grown in BG-11 medium until $Nt = 1 \times 10^5$ cells and afterwards exposed to TBT. For the set 2 controls, 26 aliquots of 1×10^5 cells from the same parental population growing in BG-11 medium were separately transferred to culture flasks containing TBT. Cultures were observed for 60 d (thereby insuring that one mutant cell could generate enough progeny to be detected), and the resistant cells in each culture (in set 1 and set 2) were counted. The cell count was performed by at least two independent observers.

Two different results can be found in the set 1 experiment, each of them being interpreted as the independent consequence of two different phenomena of adaptation. In the first case (Figure 1, set 1A), the variance in the number of cells per culture could be found to be low if resistant cells arose by physiological adaptation or specific post-selective mutations. Because every cell is likely to have the same chance of developing resistance, inter-culture (flask-to-flask) variation would be consistent with the Poisson model (variance/mean = 1). On the contrary, if high variation in the inter-

culture number of resistant cells is found (variance/mean > 1), it means that resistant cells appeared by random mutations occurring before selection, and the flask-to-flask variation would not be consistent with the Poisson model. Resistant-mutants occurred during the time in which the cultures reached N_t from N_0 cells, prior to TBT exposure (Figure 1, set 1B). The set 2 cultures serve as the experimental control of the fluctuation analysis (Figure 1). Variance is expected to be low, because set 2 samples the variance of the parental population. Thus, if a similar variance/mean ratio between set 1 and set 2 is found, resistant cells arose induced during the exposure to the TBT. If the variance/mean ratio of set 1 is significantly greater than the variance/mean ratio of set 2 (fluctuation), resistant cells arose by rare mutations that occurred before exposure to the TBT.

The fluctuation analysis also allows estimation of the rate of appearance of resistant cells. The proportion of set 1 cultures showing non-resistant cells after TBT exposure (P_0 estimator) was used to calculate the mutation rate (μ) as follows:

$$P_0 = e^{-\mu (N_t - N_0)} \quad (23)$$

where P_0 is the proportion of cultures showing no resistant cells, N_0 and N_t are the initial and the final cell number respectively and μ is the mutation rates (in mutants per cell division).

If the mutation from a normal wild-type TBT-sensitive allele to a TBT-resistant allele is recurrent, and the TBT-resistant allele is detrimental to fitness in the absence of TBT, then new mutants arise in each generation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance (21). At any one time there will be a certain number of resistant mutants that are not yet eliminated. The balance between m and the rate of selective elimination (s) will determine the average number of such mutants:

$$q = \mu / (\mu + s) \quad (24)$$

where q is the frequency of the TBT-resistant allele and s is the coefficient of selection against TBT-resistant allele calculated as follows:

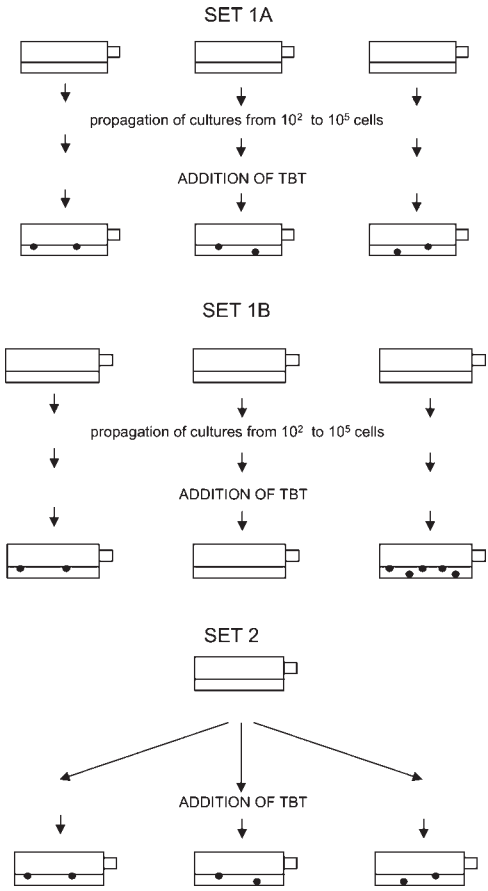

Figure 1. Schematic diagram of possible results obtained in the experiment (modified from the classic Luria-Delbrück fluctuation analysis). In the set 1 experiment, 100 different cultures of *Dc1M* strain (each started from inoculums of N_0 = 10^2 cells) were propagated under non-selective conditions (i.e. without TBT) until a very high cell density (N_t = 10^5 cells) was reached, and then TBT was added. **Set 1A:** the resistant cells arose in response to TBT during TBT exposure. In this case, the number of resistant cells in all the cultures must be similar. **Set 1B:** the resistant cells arose by rare spontaneous mutations. Most of these mutations spontaneously arose during the period of the propagation of cultures before to TBT exposure. One mutational event occurred late in the propagation of culture 1 (therefore, the density of TBT-resistant cells found is low) and early in the propagation of culture 3 (thus, density of TBT-resistant cells found is higher than in culture 1); no mutational events occurred in culture 2. In this case, the number of resistant cells in all the cultures must be different. **Set 2** samples the variance of parental populations as an experimental control. In this case, the number of resistant cells in all the cultures must be similar.

$$s = 1- (m_{TBT}{}^{r}/m_{TBT}{}^{s})$$

where $m_{TBT}{}^{r}$ and $m_{TBT}{}^{s}$ are the Malthusian fitness of TBT-resistant and TBT-sensitive cells measured in non-selective conditions (i.e. BG-11 medium), respectively.

2.5. Growth of TBT-sensitive and TBT-resistant cells on antifouling paint

Bottom of 6 wells-dish plates (Nunclon Delta Surface, Danmark) was painted with TBT- antifouling paint (Hempel, Polinya, Barcelona, Spain). Twenty-four hours latter, cell inoculums of 5×10^5 wild type TBT-sensitive cells or TBT-resistant mutants were respectively transferred to antifouling-painted plates with 10 ml of BG-11 fresh medium. Three plates of sensitive and three plates of resistant cells were maintained at 20º C under continuous light of 60 mol photom m^{-2} s^{-1} over the waveband 400-700 nm. Cultures were observed for 15 days and the final number of cells was counted using a Uriglass chambers and an inverted microscope (Zeiss Axiovert 35).

3. RESULTS

3.1. Microalgae of biofouling recovering the underwater body of ships

Numerous coastal and oceanic microalgae species proliferates on bottoms of ships directly on TBT antifouling paint (Figure 2). Most abundant species were: i) diatoms (Bacillariophyta) from genus *Navicula*, *Nitzschia*, *Asterionella*, *Amphora*, *Chaetoceros*, *Thalassiosiras* and *Rhizosolenia;* ii) Dinoflagellates from genus *Prorocentrum*, *Scrippsiella* and *Amphidinium;* iii) Haptophyta from genus *Primnesium* and *Chrysochromulina;* iv) Chlorophyta from genus *Tetraselmis* and *Dunaliella;* v) cyanobacteria (Cyanophyta) from genus *Synechococcus;* vi) Cloroxibacteria from genus *Prochloron*. These microalgae were viable in spite of their contact with the antifouling paint. In addition, resting phases (i.e. dinoflagellates cyst, diatom spores) and early stages of macroalgae (i.e. *Fucus*, *Laminaria*, *Chondrus*, *Codium* and others) were also observed.

Toxin-producing microalgae (i.e. okadaic acid producing dinoflagellate *Prorocentrum lima;* domoic acid producing diatom *Pseudonizchia pungens*) and other harmful microalgae (i.e. *Crysochromulina*) were also found attached to the bottoms of the sampled ships.

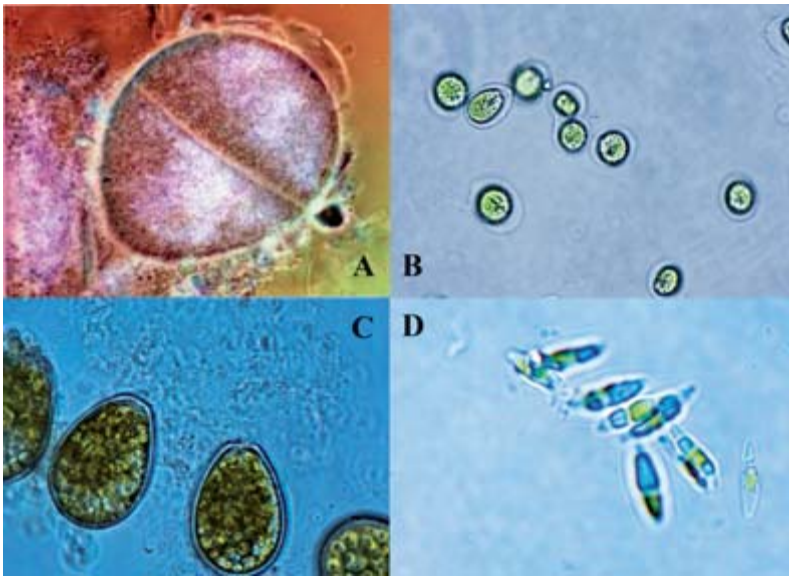

Figure 2. Example of algae of biofouling recovering the underwater body of ships. **A.** Early stage of *Fucus* (Phaeophyta); **B.** *Tetraselmis sp.* (Chlorophyta); **C.** DSP toxic dinoflagellate *Prorocentrum lima* (okadaic acid producing); **D.** pennate diatoms (*Pseudonitzschia sp*).

3.2. Toxic effect of TBT on cells of Dc1M

TBT was extremely toxic for *Dc1M* cells of wild-type genotype. Growth of *Dc1M* cells was irreversible inhibited by TBT concentrations of 10 ppm. Monitoring changes in photosynthetic performance also assessed the toxic effect of TBT. Only 10 ppm TBT were able to inhibit 79,51% of Φ_{PSII} whereas 30 ppm TBT totally inhibited photosynthesis.

3.3. Fluctuation analysis

When conducting the fluctuation analysis, the cell density of strain *Dc1* was drastically reduced in each experimental culture due to destruction of wild-type cells, by the toxic effect of TBT. However, after further incubation for 60 days, some cultures increased in density again, apparently due to growth of a TBT-resistant variant.

In the case of set 1, only some cultures recovered after 60 days under TBT exposure (Table 1). By contrast, every set 2 culture recovered, indicating the presence of TBT-resistant cells in all cultures. A high fluctuation (variation) in the set 1 experiment (from 0 to more than 10^5 resistant cells per culture flask) was found (Table 1). In contrast, in set 2 controls all the culture flaks showed from 10^4 to 10^5 TBT-resistant cells (a scarce variation due to experimental error), which indicated that the high fluctuation found in set 1 cultures should be due to processes other than sampling error. The fluctuation of set 1 experiment indicates that TBT-resistant cells arose by rare, pre-selective spontaneous mutations rather than by specific adaptation in response to TBT.

Table 1. **Fluctuation analysis from TBT-sensitivity to TBT-resistance in *Dc1M* strain**

	Set 1 experiments	**Set 2 controls**
No. of replicate cultures	96	26
No. of cultures containing the following no. of TBT resistant cells:		
0	65	0
from 1 to 10^3	5	0
from 10^3 to 10^4	7	0
from 10^4 to 10^5	8	26
more than 10^5	11	0
Fluctuation	yes	
μ (mutants per cell per generation)	7.071×10^{-8}	

The estimated mutation rate (μ) from TBT-sensitivity to TBT-resistance in *Dc1M* strain was 7.07×10^{-8} mutants per cell per cell

division. The Malthusian parameter of fitness of TBT-resistant mutants and wild type sensitive cells (0.085 and 0.24 respectively) were used to estimate the coefficient of selection of TBT-resistant mutants (s). By using the values of m and s, the frequency (q) of resistant alleles was estimated in *circa* 3 TBT-resistant mutants per 10^{-4} wild type sensitive cells.

3.4. Growth of TBT-sensitive and TBT-resistant cells on antifouling paint

When wild type sensitive *Dc1M cells* were grown during 15 days on antifouling paint in Nunclon wells-dish plates a massive destruction of cells was observed. Antifouling paint totally inhibited the cell growth and most of the cells were destroyed (initial no. = 5.0×10^5 cells ml^{-1}; final no. = 1.6×10^5 cells ml^{-1}). In contrast TBT-resistant mutants were able to grow directly on antifouling paint (initial no. = 5.0×10^5 cells ml^{-1}; final no. = $1,5 \times 10^6$ cells ml^{-1}).

4. DISCUSSION

Numerous microalgae were found in the biofouling recovering the underwater body of ships. As expected pennate diatom species, which can easily be attached to surfaces by mucilage secretions (25) are very numerous. Also benthic dinoflagellates are abundant in the ships biofouling. Obviously, early stages of macroalgae easily colonize biofouling.

A lot of resting stages of phytoplankton (cysts and spores) were found in the biofouling. These resting phases seems to be especially well adapted to survive in the biofouling. Since most phytoplanktonic organisms has spores and resting cysts that serve as a perennation function (25), i.e. they allow phytoplankton to survive periods that are not suitable for growth and afterwards germinate when conditions improve, biofouling is a potential source for microalgae dispersion.

Apparently, several species detected on ships biofouling could be exotic species. Taken in account that different families of clones

maintained by asexual growth constitute the population genetic structure of many phytoplankton species (26), also exotic genotypes cold arrive to far regions via biofouling. Some of these non-indigenous introduced species or genotypes could proliferate in remote areas and become invasive species.

Long-distance transport of toxin-producing microalgae it is particularly worrisome. Three harmful microalgae species (*Prorocentrum lima, Pseudonitzchia pungens* and *Crysochromulina polilepis* implicated in DSP, ASP and mass mortalities of fish respectively) were detected in a small area (less of 3 cm^2) of biofouling in one boat. This provides robust evidence on the magnitude of biofouling-associated transport problem. During the 1950s Margalef (one of the best phytoplankton taxonomists) analyzed the microalgae species composition in the Ría de Vigo, NW of Spain, (27-29) providing extensive list of phytoplankton species composition. However, Margalef never detected several microalgae species that constitutes the main problem of harmful algal blooms (HABs) in the Ría de Vigo nowadays. Could have arrived recently these algae to Ría of Vigo via ships biofouling? Since Ría of Vigo with other Rías of NW of Spain (i.e. Pontevedra, Arosa, Muros and Lorbé) is the most important area for shellfish aquaculture in European Union (i.e. mussels, cockles, oysters, clams and scallops) the economic impact of HABs species introduction of could be enormous.

The key to explain microalgae species transport via ships biofouling is know the mechanisms that allow to these species to survive long time attached to antifouling paint. Apparently, adaptation to biofouling paints is not easy. Growth and photosynthetic performance of the experimental strain *Dc1M* was irreversible inhibited by TBT concentrations many times lower than those used in antifouling paints. Antifouling paint on the bottom of Nunclon wells-dish plates rapidly destroyed wild type *Dc1M* cells. Since adaptation to antifouling paints seems to be difficult, the classic evolutionary point of view assumes that genetic adaptation at such extreme conditions is a gradual process (reviewed in 30).

In contrast, here we propose an alternative explanation for adaptation of microalgae to antifouling paints. When the experimental strain *Dc1M* was cultured in TBT, usually cultures show massive destruction of the sensitive cells by the toxic effect of TBT. However,

after further incubation for 60 days, some cultures became increased in density again, due to the growth of cells that were resistant to the toxic effect of TBT.

The approach for understanding adaptation of microalgae to TBT is to analyze the rare variants that proliferate after the massive destruction of the sensitive cells by this selective agent. Fluctuation analysis is the appropriate procedure to discriminate between TBT-resistant cells arising by rare spontaneous mutations occurring randomly during replication of organisms prior to exposure to TBT and TBT-resistant cells arising through specifically acquired adaptation induced by TBT (reviewed in 31).

Genetic adaptation by rare spontaneous mutation is the mechanism allowing adaptation of microalgae to TBT contamination. The large fluctuation in number of resistant cells detected in the set 1 experiment in contrast to the scarce fluctuation in set 2 controls, unequivocally demonstrates that these resistant cells arose by rare spontaneous single mutations (which mainly occur prior to TBT exposure) and not through direct and specific adaptation in response to TBT. Results of fluctuation suggest that only one gene is implicated in the TBT-resistance process. If several genes are involved in TBT resistance then we should be unable to detect TBT-resistant cells growing in flaks of set 1 experiment because the scarce probability of occurrence for several mutations at the same time. Consequently, microalgae can adapt to antifouling paints much more rapidly by single mutations that if the ability to survive require multiple mutations. Several evidences suggest that single spontaneous mutation at one locus can achieve adaptation of mesophile microalgae to other severe anthropogenic contaminants including antibiotics (32, 33), herbicides (34-36) other potent biocides (37), and crude oil (38, 39). Even, microalgae are able to rapid adaptation to heavy metals (which are the toxic compounds of antifouling paints) by mean of single pre-selective mutations (40-43). Resistant mutants of microalgae are usually used to manufacture specific biosensors (44, 45). This capability is perhaps a consequence of microalgae ability for adaptation to hostile natural environments by mechanisms based on single mutations (46-50).

The *DcM1* strain was widely used to measure mutation rates from sensitivity to resistance to several anthropogenic and natural

contaminants (summarized in Table 2). A remarkable feature was that mutation rate from TBT-sensitivity to TBT-resistance ($\mu = 7.07 \times 10^{-8}$ mutants per cell per cell division) was two or three orders of magnitude lower than those we have described for resistance to other biocides in *Dc1M* strain. It may be that the acquisition of TBT-resistance exemplified by the present work is intrinsically more rare than other kinds of mutation. However, this aspect remains to be investigated.

Table 2. **Mutation rates (μ) in mutants per cell division of *Dc1M* strain from sensibility to resistance against different lethal anthropogenic and natural contaminants**

Selective agent	μ	source
TBT	7.07×10^{-8}	this paper
DCMU	2.1×10^{-6}	Costas *et al.* (2001) (34)
TNT	1.4×10^{-5}	García-Villada *et al.* (2002) (37)
Formaldehyde	3.6×10^{-6}	López-Rodas *et al.* (2008c)
Tinto River	1.4×10^{-6}	Costas *et al.* (2007) (49)
Geothermal waters	1.4×10^{-6} to 1.5×10^{-5}	Costas *et al.* (2008) (47)
Mynydd Parys pond	1.6×10^{-6}	López-Rodas *et al.* (2008a) (43)
Aguas Agrias stream	1.1×10^{-6}	López-Rodas *et al.* (2008b) (48)
Vulcano Island pond	4.7×10^{-7}	López-Rodas *et al.* (2009) (46)

Mutation from TBT-sensitivity to TBT-resistance occurs recurrently. However, TBT-resistant cells have diminished fitness compared to wild type TBT-sensitive cells. Consequently, the balance between the recurrent appearance of mutants by rare pre-selective mutation and their elimination by natural selection controls the presence of TBT-resistant cells in the populations. As a result, an equilibrium frequency of around 3 TBT-resistant mutants per each 10^{-4} wild type sensitive cells should maintain in *Dc1M* algal populations non-exposed to TBT. Taking into account both the relatively high number of resistance-mutants and the countless cells comprising algal populations, it could be hypothesized that algal colonization of antifouling paints should be almost instantaneous because TBT-resistant cells are always present in microalgae

populations. The experiment of colonization of Nunclon wells-dish plates painted with antifouling demonstrates validity of this hypothesis.

Long-distances navigation is a characteristic of human civilizations. A lot of microalgae stowaways invade the underwater body of our ships to disperse worldwide. Since microalgal toxins are among the most important emerging health risk due to shellfish consumption (51-53) introduced toxic microalgal species are an important menace.

5. CONCLUSION

1. Marine ecosystems are specially affected by toxin-producing introduced species of microalgae with devastating impacts on aquaculture, fishery, tourism and public health (economic cost estimated in $ billions). Although ships ballast water has been considered the major vector in dispersion of microalgae, numerous species of microalgae travel great distances forming the biofouling attached the underwater body of ships.
2. In spite of toxic antifouling paints recovering the bottom of ships microalgal species are resistant to these antifouling.
3. Antifouling paint-resistant microalgae arose by rare mutation, which occurs spontaneously in natural populations prior to antifouling paint exposure. The balance between the recurrent appearance of antifouling-resistant mutants and their elimination by natural selection controls the presence of antifouling-resistant cells in the natural populations. An equilibrium frequency estimated in around 3 antifouling resistant mutants per each 10^{-4} sensitive cells should assure a rapid microalgal colonization of the underwater body of ships.

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