Chapter 4

SYNERGISM BETWEEN ANTIFOULING BIOCIDES

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4.1 INTRODUCTION

A major advance in the control of fouling by marine algae on the underwater parts of ships was made in the early seventies with the development of a type of antifouling paint known as the copolymer system (see Evans, 1981). Unlike conventional systems, which are physical mixes releasing toxin (eg. cuprous oxide) at a diminishing rate and therefore at reduced effectiveness with time, the copolymer system is a chemical mix of two monomers. A film-forming paint-resin ("binder") is made by the vinyl copolymerisation of methyl methacrylate and tributyltin methacrylate. This copolymer contains a high molar proportion of toxic organotin moieties, linked by ester bonds to the polymer backbone (Evans, 1970). The copolymer hydrolyses in seawater to release the principle biocide, a triorganotin such as tributyltin oxide, in a controlled manner. Other biocides (pigments) such as cuprous oxide, may also be incorporated as physical mixes into the polymer matrix and are released as hydrolysis occurs. In addition, the surface in itself is self-polishing, i.e. surface roughness decreases as the polymer is eroded, so that the composition is known as self-polishing copolymer (SPC). The rate of polishing is dependent upon polymer composition (proportions of the monomers), vessel speed, water temperature, pH and salinity.

Although SPC systems effectively control the larger fouling algae, they are not effective in the complete control of slime-forming organisms (French, Evans and Dalley, 1985). Movement of the ship through the water is essential for optimal self-polishing and when the polishing rate is not optimal, microscopical biological slimes occur which although visually insignificant contribute markedly to the frictional resistance of a moving ship (Lewthwaite et al., 1985). Slime-covered surfaces also provide a natural substrate for settlement of macroalgal spores, eg. *Ectocarpus*, which may germinate and grow intermixed with it. In addition, slime may retard the rate of copolymer hydrolysis and hence biocide release (leaching) from the paint. Biological slime films are composed of an aggregation of organisms, chiefly bacteria and diatoms, held in a semi-rigid mucilage. The predominant diatoms on SPC paints belong to the genera *Amphora*, *Amphiprora*, *Achnanthes*, *Stauroneis* and *Navicula* (Callow, 1986; French and Evans, 1986).

Since under average ship-operating conditions, slime films dominated in particular by the diatom *Amphora* develop on SPC paints,research has been undertaken aimed at finding (1) new biocides which may be added to such paints to give better slime control and, (2) biocide combinations which in acting together will give improved control and permit the use of reduced triorganotin levels. Here we report on the results of an investigation into the effects of tributyltin oxide (TBTO), together with two cationic surfactants, 1-dodecylguanidine acetate (1-guanidinododecane acetate; trade names dodine, cyprex, melprex) or 2-dodecylguanidine acetate (2-guanidinododecane acetate; guanidine, (1-methylundecyl)-monoacetate) on the diatom *Amphora coffeaeformis*. In the interests of brevity the two latter compounds will be referred to as 1-DGA and 2-DGA respectively in this communication.

4.2 MATERIALS AND METHODS

Axenic stock cultures of *Amphora coffeaeformis* var. *perpusilla* (Grunnow) Cleve derived from a clonal culture as described by Blunn and Evans (1981) were maintained at 20^{o}C in 2dm^3 flasks of Guillard's F2 medium (Guillard and Ryther, 1962), as described by Callow and Evans (1981). The medium was prepared with Instant Ocean (Aquarium Systems, U.S.A.) artificial seawater.

The effects of biocides, singly or in combination, were determined as follows: 10 cm^3 log phase culture and 90cm^3 sterile F2 medium were transferred aseptically to 250cm^3 Ehrlenmeyer flasks which were maintained on an orbital shaker (180 rpm) at 20^{o}C under continuous irradiance at a photon flux density of 130 uE m^{-2}s^{-1}. After 24h, biocidal solutions were added to give the desired concentrations. Four replicates per treatment were used.

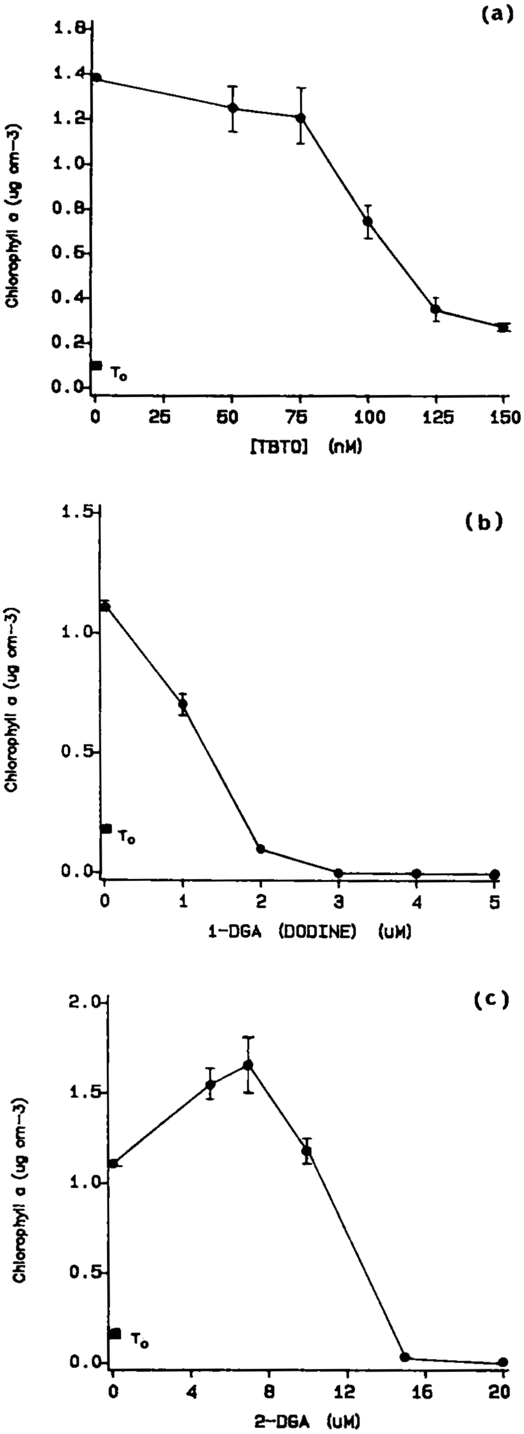
Stock solutions of TBTO (Aldrich Chemicals) in ethanol were made up at x1000 final concentration and stored in the dark at 4°C. Controls contained 0.1% ethanol. Stock solutions of 1-DGA (Cyanamid) and 2-DGA (synthesised by the Coalite Group plc, Chesterfield, Derbyshire) were freshly made up at X1000 final concentration in ethanol. Controls contained 0.1% ethanol, although this had no effect on growth.

Chlorophyll$_a$ concentrations were determined 96h after addition of biocides. After dislodging adhering cells, 10cm^3 of cultures were filtered onto cellulose nitrate membrane filters pore size 1.2 uM, which were then immersed in 5 cm^3 dimethyl sulphoxide (DMSO) (Shoaf and Lium, 1976). After 1h (in darkness) tubes containing the dissolved filters were vortexed and left for a further 30min to allow cells to settle. The absorbances of the cell-free supernatants were then determined at 630 and 664nm using a LKB Ultrospec 4050 spectrophotometer, and chlorophyll$_a$ concentration determined using the equation of Holden (1976). The means of the replicates were calculated and the standard errors are shown in the figures.

4.3 RESULTS

TBTO exhibits pronounced algicidal activity against the diatom A. coffeaeformis and a total kill is achieved at a concentration of 250nM. Its LC-50 value is approximately 100nM (Fig.4.1a) i.e. at this concentration the growth rate (determined by reduction in chlorophyll concentration) is reduced by 50%, relative to the controls. 1-DGA and 2-DGA also showed significant algicidal action against A. coffeaeformis, giving LC50 values of 1.25 uM (Fig.4.1b) and 13.0 uM (Fig.4.1c) respectively.

In order to determine whether synergism is occurring, i.e. whether two biocides used together give an effect greater than the sum of their individual effects, it is necessary to use biocide concentrations which individually cause not more than approximately 25% inhibition. Used at such concentrations, both 1-DGA (Fig.4.1d) and 2-DGA (Fig.4.1e) showed a pronounced synergistic effect when used in combination with TBTO, the latter giving the more marked response. Thus, for example, inhibition of growth by 10nM TBTO on its own is not significantly different from the control and inhibition by 1.3uM 1-DGA used singly is


(a)
Chlorophyll a (ug cm-3)
1.8
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
To
0
25
50
75
100
125
150
[TBTO] (nM)
(b)
Chlorophyll a (ug cm-3)
1.5
1.0
0.5
0.0
To
0
1
2
3
4
5
1-DGA (DODINE) (uM)
(c)
Chlorophyll a (ug cm-3)
2.0
1.5
1.0
0.5
0.0
To
0
4
8
12
16
20
2-DGA (uM)

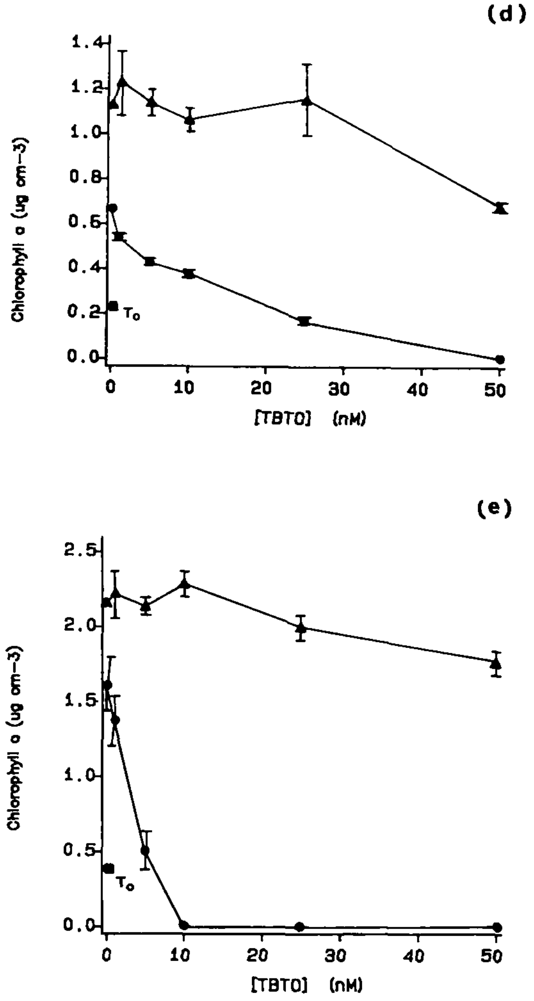


Fig.4.1. The effects of various biocides on chlorophyll$_a$ levels in *A. coffeaeformis*. (a) tributyltin oxide (LC$_{50}$ 104nM), (b) 1-dodecylguanidine acetate (LC$_{50}$ 1.25uM), (c) 2-dodecylguanidine acetate (LC$_{50}$ 13.0uM), (d) tributyltin oxide (▲), tributyltin oxide + 1.3uM 1-dodecylguanidine acetate (●), (e) tributyltin oxide (▲), tributyltin oxide + 8.0uM 2-dodecylguanidine acetate (●). T$_0$= chlorophyll concentration when biocide added. Ethanol control = same as zero TBTO.

approximately 40% compared with the control (Fig.4.1d), but when used together at these concentrations they inhibit by some 65% (Fig.4.1d). Using 2-DGA and TBTO the synergistic effect is even more pronounced. 8uM 2-DGA gave 25% inhibition and used singly, 10nM TBTO gave no inhibition (Fig.4.1e). However, 100% inhibition was observed when the two compounds were used together at these concentrations (Fig.4.1e). With 1-DGA on the other hand (Fig.4.1d), a TBTO concentration of 50nM was required, with 1.3uM 1-DGA, to give 100% inhibition.

4.4 DISCUSSION

Although 1-DGA on its own (LC50 1.25uM) is more toxic to *A. coffeaeformis* than 2-DGA (LC50 13.0uM), the results clearly demonstrate that in laboratory experiments, the greatest reduction in TBTO concentration can be made (about 50-fold), without loss of algicidal activity, if 2-DGA is present. If the flux of TBTO and 2-DGA could be maintained at synergistically effective levels within the boundary layer of an SPC antifouling paint system, the tin level of the paint could in theory be considerably reduced, whilst still retaining very effective control with respect to the fouling diatom *A. coffeaeformis*. Whether such control of this and/or other fouling diatoms on paint surfaces would occur in practice is not known.

1-DGA is a fungicide with cationic surface active properties which has been used particularly against scab of apple and pear since its introduction in 1956. Its toxicity appears to result from its effects on cell permeability and on mitochondrial and/or chloroplastic electron transfer. In *Saccharomyces pastorianus* Brown and Sisler (1960) found that it exerts its toxic effect by altering the permeability of the cell, resulting in loss of vital cellular constituents, and by inactivating certain vital enzymes. Amongst a range of oligoamines and oligoguanidines tested by Srivastava and Smith (1982), monoguanidines, including 1-DGA, were the most toxic in their effect on higher plant membranes, causing massive efflux of betacyanin from beetroot discs and ion efflux from swede discs. Hassall (1982) also reported that 1-DGA acts primarily by disrupting cell membranes, the hydrophilic part of the molecule being suggested to dissolve in the membrane lipid and the hydrophobic part to alter the attachment of membrane proteins. In addition to altering cell permeability, there is evidence that guanidines, including 1-DGA, inhibit energy transfer in mitochondrial systems in vitro (Pressman, 1963). Mottley (1978) also found good evidence that n-dodecylguanidine (and n-octylguanidine) affected state 3 (ADP stimulated) non cyclic electron transport in pea chloroplasts, and inhibited Ca^{2+}-dependent ATPase activity in isolated chloroplast fragments of the unicellular green alga Chlamydomonas reinhardi. In pea chloroplasts, energy transfer inhibition occurred at low concentrations, uncoupling of electron transport from photophosphorylation at intermediate concentrations, and direct inhibition of electron transport at relatively high concentrations (Mottley, 1978).

The first recorded use of 2-DGA was in 1973, as a fungicide for seeds (Reckitt and Colman Products Ltd. Brit. Appl. 73/40, 168, 24 August 1973) and wood preservation (Reckitt and Colman Products Ltd. Brit. Appl. 73/40, 169, 24 August 1973). The guanidine salt 2-guanidinododecane phosphate has also been used as a seed fungicide (Brit. Appl. 73/40, 168 above), as well as in the control of fungi on potato and tomato (Reckitt and Colman Products Ltd. Brit. Appl. 73/40, 166, 24 August 1973) and in the inhibition of fungal growth when incorporated into a paint composition (Springle, W.R. and Rushman, D.F. Brit. Appl. 40,305/73, 24 August 1973). The phosphate salt was also recorded as having a wide spectrum of activity against algae, fungi, bacteria and lichens (Rushman, D.F., Reckitt and Colman Products Ltd. Brit. 1,475,073 (Cl. C07 Cl 29/12) 1 June 1977, Appl. 73/40, 137, 24 August 1973). More recently, guanidinoalkanes such as guanidinododecane acetate have been rendered soluble by combining with metal chelates, so facilitating their formulation and improving their efficiency as fungicides (Everest-Todd, S., Wheatley Chemical Co. Ltd. GB Appl. 82/29, 425, 14 October 1982). The literature is thus confined to reports relating to investigations on 2-DGA as a fungicide in respect of patents, and there is as yet no work on the mode of action of this compound. However, it is considered likely that its cellular effects are not dis-similar to those of 1-DGA. The stimulation of growth caused by low concentrations of 2-DGA (Fig.1c) suggest an initial increased permeability to nutrients, without metabolic damage. However, the inhibitory response on growth above 7uM clearly suggests cellular damage.

The biocidal effects of organotins are well-documented. In the algae, Mottley (1978) reported that several triorganotins inhibited Ca^{2+}-dependent ATPase activity in C. reinhardi chloroplasts, and in the multicellular green algae Enteromorpha intestinalis and Ulothrix pseudoflacca Millner and Evans (1980) showed that triphenyltin chloride (TPTC) acts as a energy transfer inhibitor of both respiration and photosynthesis.

There are many examples of synergism between biocides and this has been used to enhance the action of drugs, antibiotics and fungicides (e.g. Albert, 1973; Shadomy, 1977; Goss and Marshall, 1985). Synergism between biocides has also been used to advantage in formulations of antifungal paints (e.g. Rusch, 1978; Lloyd, 1980). As far as is known, there has been no basic study on the use of a triorganotin and 1- or 2-DGA together.

The mechanisms underlying the pronounced synergistic responses seen in the present investigation can be speculated upon. Uptake of radio-labelled TPTC has been found to be essentially a passive process (Millner and Evans, 1981), the rate being proportional to TPTC concentration. It can therefore be postulated that the increase in membrane permeability resulting from the presence of 2-DGA allows more TBTO to enter the cell, and the elevated intracellular concentration will result in enhanced inhibition of energy transfer even at low exogenous concentrations of TBTO. Alternatively, or in addition to this, 2-DGA may increase the affinity for TBTO of the specific binding sites in the thylakoid membranes, or itself inhibit for example Ca^{2+}-dependent ATPase activity. Experiments are now necessary to test these possibilities. These would include monitoring the uptake of radio-labelled TBTO by A. coffeaeformis cells in the presence of 2-DGA at different concentrations, and in its absence; measurements of leakage of ions from cells in the presence of 2-DGA, and a detailed metabolic study of the effects of 2-DGA +/- TBTO on the respiratory and photosynthetic pathways.

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