MARINE CHEMICAL ECOLOGY : CHEMICALS SPEAK SOFTLY IN ALL LANGUAGES

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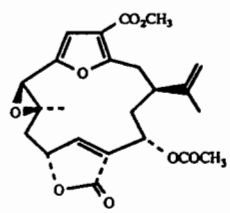
In today's lecture I'd like to describe a day in the life of a soft coral from the perspective of the marine chemical ecologist. The coral whose life story I'll focus on is a very well known and much studied soft coral called *Sinularia flexibilis*. *S. flexibilis* is probably one of the most common soft corals in the Indo-Pacific. Indeed almost any natural products group which has made collections in this region, has at one time or another extracted and studied the chemistry of *S. flexibilis*. In order to tell the story of *S. flexibilis* it is necessary to go back to the beginning, and like other soft corals, *S. flexibilis* is a dioecious species in which some colonies contain all male polyps, while other colonies contain all female polyps. This means that a given colony will release eggs or sperm into the water and these different types of gametes must meet in this vast oceanic soup. From the very minute that the eggs are released into the water column, they are already under severe pressure for survival. Groups of fish swarm around the colony and consume vast qualities of the eggs on the night of spawning. Fortunately for the soft corals, kind hearted organic chemists often come along and put nets over the female colonies and protect the eggs from fish predation. In this way, an organic chemist can collect several grams of eggs from a given soft coral. By using this technique, we know that we've trapped the eggs of one and only one species (1).

The first time we looked at the chemistry of the eggs of a *Sinularian* soft coral, we found two quite well known compounds in the eggs, pukalide 1 and epoxypukalide 2. Pukalide was first isolated from soft coral *Sinularia abrupta* by the Scheuer group in about 1975. The colony from which the eggs were derived, contained virtually none of the two compounds prior to spawning, while the eggs were exceedingly rich in these metabolites. Indeed within a week or two of spawning, there was no trace of these compounds in the extract of the colony. Within a few days of the eggs being released and fertilised, they develop into what are called planulae, a mobile form of these corals, and these planulae drift or swim in the water column until they are ready to settle. The interesting thing we determined was that the chemical composition of the eggs included significant amounts of *egg-specific compounds*, compounds like pukalide and others. The planulae, within a few days of spawning, showed absolutely no trace of these same compounds, and yet other diterpenes which were present in the eggs were still retained (2). We studied the eggs from a number of different species, and concluded that almost all the eggs of soft corals contain *egg specific compounds* ie compounds which were either absent or only present at very low levels within the colony releasing them. In the eggs, they were often 10% of the dry weight ie at very high concentrations. One of these compounds in a *Lobophytum* species was the acetoxy methylester of prostaglandin A_2 3. We knew that prostaglandins have an effect on smooth muscle, and we knew that all these compounds, pukalide 1, thunbergol 4 and many others are present in the eggs for only a short time after release. It therefore seemed likely that

their role had something to do with the ovulation process, the process of egg release from the polyps.

I was able to coerse a couple of my physiologist friends, Dr Mike Capra from Queensland University of Technology and Dr Mike Pass from the University of Queensland to carry out some experiments in which they exposed isolated polyps from a soft coral to different concentrations of these egg specific compounds, pukalide 1, the prostaglandin 3, sarcophytoxide 5 and others. It was found that each of these compounds had quite a dramatic effect on the smooth muscles of these polyps ie the polyps contracted quite strongly (3). It seems likely then that these compounds are implicated in the processes involved in the release of eggs from the polyps.

pukalide



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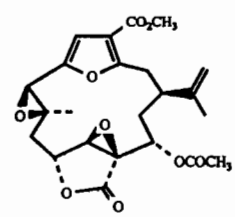
epoxypukalide



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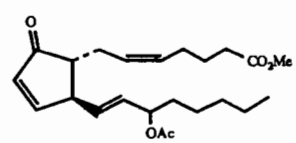

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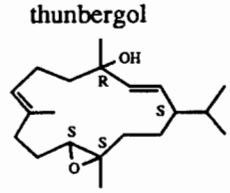
thunbergol



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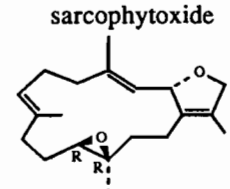
sarcophytoxide



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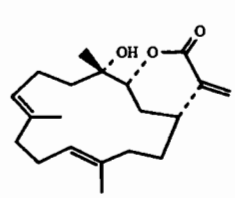
Uchio's toxin



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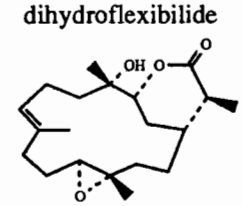
dihydroflexibilide



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The planulae that we spoke about earlier contained virtually no *egg specific compounds* after five days and usually settle on the underside of solid surfaces, gradually turning into what is identifiable as an octocoral polyp : a polyp that possesses eight pinnate tentacles around the mouth. Ultimately this single polyp divides into two, the two into four etc, and this process ultimately produces a mature colony of *Sinularia flexibilis*. After that, once an adult colony has formed, these colonies may divide asexually to form two separate colonies, each of which goes away to carry on its existence in a normal manner. The interesting thing about *S. flexibilis*, is that it is an extraordinarily fleshy soft coral. The polyps are exceedingly vulnerable to fish predation and yet there is very little fish predation on *S. flexibilis*, an indication that it contains chemistry which helps it to survive.

Over the years we have studied the feeding deterrent and the ichthyotoxic properties of this and other soft corals. We studied the ichthyotoxicity of freshwater macerates of the soft coral, which we spin down to separate the spicules and coral tissue from the supernatent liquid. This is regarded as an aqueous extract of the coral. We tested these aqueous extracts of a number of soft corals, and found that approximately half of the extracts were acutely toxic to the mosquito fish, *Gambusia affinis*, while the other half are not particularly toxic, ie almost totally benign (4).

Sinularia flexibilis contains a number of cembranolide diterpenes which possess considerable biological activity. In fact, of the metabolites in *S. flexibilis*, the compound which we call Uchio's toxin 6 is a very minor component, only detectible and isolable by using bioactivity directed fractionation. This compound is actually a hundred or more times more toxic than either sinulariolide 7 or flexibilide 8. Uchio managed to isolate his compound using bioassays with killifish. The major components are sinulariolide 7, flexibilide 8, and dihydroflexibilide 9, and the ratio of these compounds depends on the coral. We'll come back to that issue later, but if ichthyotoxicity is important to the survival of *S. flexibilis*, what are the other compounds doing. The question is whether ichthyotoxicity accounts for the total defensive attributes of *Sinularia flexibilis*, or whether taste is also important. We carried out experiments with *Gambusia affinis* to see whether the chemical substances that are carried into the aqueous extracts of the soft coral are indeed deterrent to a generalist predator represented by *G. affinis* and found that they were quite deterrent (5).

When you assess the total situation you realise that soft corals are protected by ichthyotoxicity, and they are protected by feeding deterrents. The other form of protection relates to the distribution of limestone spicules throughout the tissue. We were interested in whether there was a correlation between the protection afforded by limestone spicules and toxicity, in other words whether spiculation was a substitute for toxicity. If colonies are very frail with very poor physical defense of the polypary like *Sinularia flexibilis*, we expected toxicity be high. If they have a highly spicule packed region close to the point where the coral attaches to the substrate like *Sinularia dura*, then the toxicity should be significantly less, and that's what has been observed in the field and laboratory (6). *Sinularia flexibilis* is highly chemically defended while *Sinularia dura* is not toxic at all. Even when you've got your whole defensive act together, it's interesting to find that some specialist predator will still get in under your defense. An example of this is *Ovula ovum*, the egg cowrie which consumes *Sinularia* colonies irrespective of their chemical defenses (7).

That really covers the aspect of soft coral defense. Most are chemically defended, which accounts for their success against predators in the field.

The other matter of great importance to soft corals, is that once you establish yourself on a reef, how do you keep other organisms from getting access to the area that you are occupying. It seems quite likely, as evidenced by this photograph taken by Betty Willis, that soft corals use chemistry to provide this competitive edge (Fig.1). This is an example of *Sinularia flexibilis* actually killing and inhibiting the growth of a scleractinian coral *Pavona cactus*. This example is quite striking. We went around the field and looked at many other examples. In order to prove that *S. flexibilis* releases chemicals into the environment and inhibits the growth of neighbours, we first had to show that soft corals could cause the death of hard corals. To do this we relocated soft coral colonies including *Sinularia*, *Lobophytum* and *Xenia* species into healthy stands of hard coral. The interesting result was that in all cases, we observed necrosis in the hard coral (8). This was statistically significant, and the effect was observed even through space, without any physical contact. What the experiment showed was that if we put a soft coral near a hard coral, it causes necrotic effects in the hard coral. The question was, is the effect caused by chemistry or by some other means.

We then wished to see if we could detect diterpenes from the soft corals in the sea water surrounding a soft coral. In order to do this, we developed a submersible sampling apparatus which comprised an acrylic enclosure and a submersible bilge pump which pumped sea water from the enclosure onto four Sep-pacs. We were able to show that there were significant amounts of the major terpenoids from the soft coral in the sea water surrounding the coral (9). The final question was do sinulariolide 7, flexibilide 8 or dehydroflexibilide 9 actually cause death in hard corals. The answer was determined in an experiment carried out at Lizard Island, where we took healthy branches of hard coral and put them in the presence of pure diterpenoid toxins. In all cases, flexibilide 8 killed the hard corals *Acropora formosa* and *Porites andrewsi* at between 5 and 10 ppm (10). We had thus shown that soft coral compounds cause death in the hard corals.

We then carried out some more detailed studies on the effect of sub lethal doses of soft coral toxins on hard corals. We wished to see the effects of the soft coral metabolites on hard corals in terms of their photosynthetic activity and respiration. The results we found were that in general these diterpenes, especially flexibilide 8, caused the respiration rate in the hard corals to increase, while causing a depression in photosynthesis (11). Then in a more recent study, Tess Aceret assessed the effect of low level concentrations of toxins on hard coral polyps and branches, and observed the physical changes which occurred in the presence of sub-lethal doses of flexibilide 8. The first effect she observed was the expulsion of viable zooxanthellae. (Fig. 2a) In other words the chemicals put stress on the system, and viable photosynthetic algae were expelled from the hard coral. The next effect was that a number of nematocysts were expelled, and the majority of these nematocysts were viable when expelled from the colony (Fig. 2b). The final effect that occurred was that the polypal activity declined noticeably under the influence of these chemicals and ultimately the coral died (12) (Fig. 2c). This series of experiments really showed that soft corals were capable of exerting an allelopathic effect on neighbouring hard corals.

It is interesting to consider the smorgasbord of compounds present in *Sinularia flexibilis* and to ask what for purpose, each of these components is there. Uchio's compound **6** was ichthyotoxic, so it provided a defensive strategy for the coral. The second compound flexibilide 8 (and possibly dehydroflexibilide 9) provided the competitive edge. They are allelopathic agents, while sinulariolide 7, in the same assay against hard corals, was completely ineffective. This suggests that sinulariolide 7 carries out some other function. Indeed a number of years ago, Ben Tursch identified that function as algicidal. We carried out some other experiments to test the antifouling role of these chemicals. We used coral skeletons as

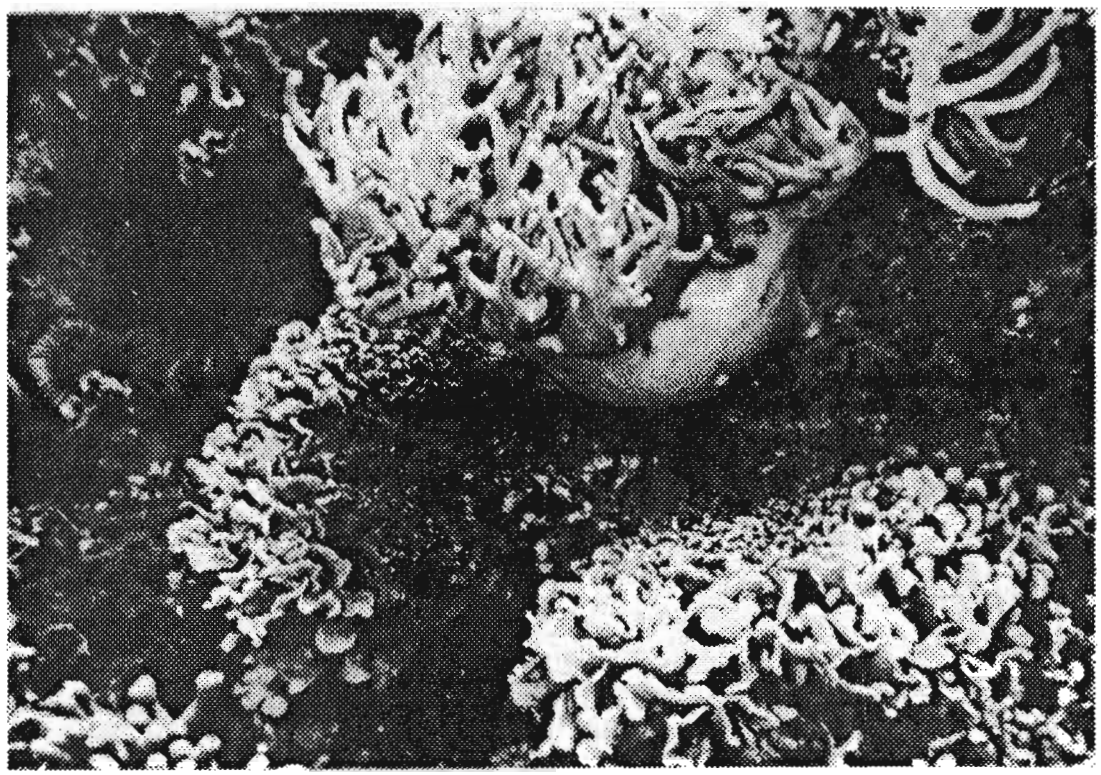
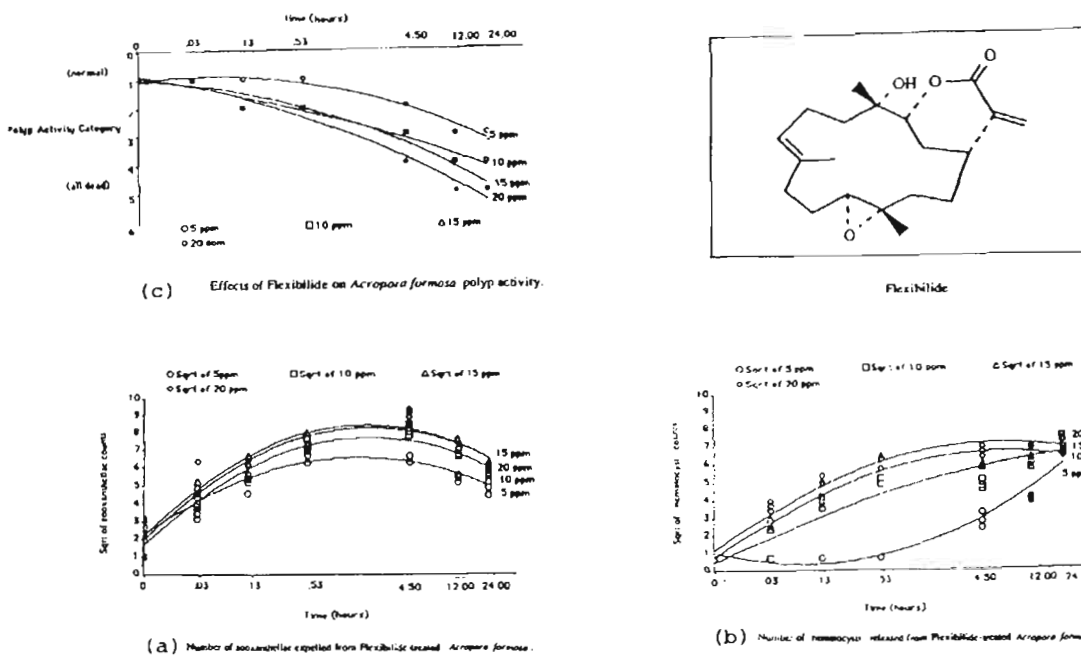


Fig. 1 : Colony of the soft coral *Sinularia flexibilis* inhibiting the growth of the hard coral *Pavona cactus* (Photo : B. Willis)

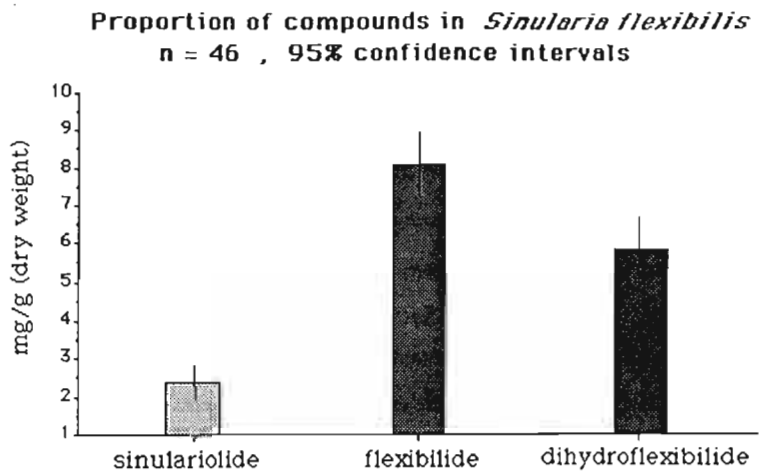


Controls (Seawater only and Seawater with Ethanol) did not show any significant effects on polyp activity, number of zooxanthellae expelled and nematocysts released

Fig. 2 : Effect of sub-lethal doses of flexibilide on *Acropora formosa*

settlement plates acting as a lid for an acrylic container. We placed an aqueous extract of *Sinularia flexibilis* and other coral extracts in the acrylic container. We determined that 50% of these metabolites leached out through the coral lid in 30 days. When we put these containers out in the field containing extracts of *S. flexibilis*, we found that algal growth on the coral lid was seriously inhibited in the treatment containers relative to the controls, which contained only seawater.

In a recent series of experiments at Lizard and Orpheus Islands, we actually looked at the ratio of sinulariolide 7, flexibilide 8, and dehydroflexibilide 9 present in colonies of *Sinularia flexibilis*. The variation was considerable, some colonies containing no sinulariolide 7 while others contained larger amounts of flexibilide 8 (Fig. 3a). The next experiments were carried out at Lizard Island and at two of three sites, our results suggest that there were indeed differences in the levels of flexibilide 8, and that these were related to the size of polypary.



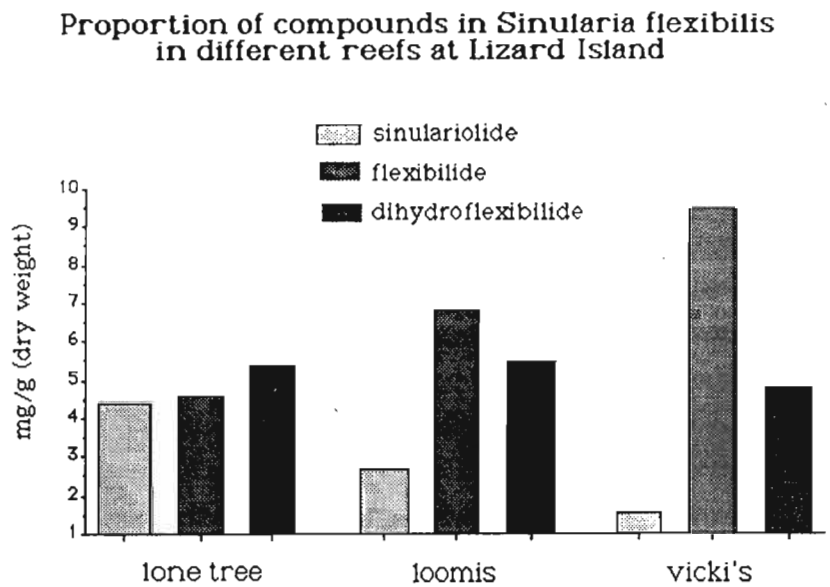


Fig. 3 : Distribution of secondary metabolites of *Sinularia flexibilis*

There was no such inverse correlation with basal size at the third site (Vicki's). The three different sites in the study mentioned above, were called "Lone Tree", "Loomis", and "Vicki's". We found that the amount of flexibilide) increased noticeably going from "Lone Tree" to "Loomis" and from "Loomis" to "Vicki's" (Fig. 3b). The difference between these sites was that "Lone Tree" was a *Porites* dominated site, while "Loomis" was a little more complex with *Porites*, *Acropora*, and a few other species being common near *S. flexibilis*. *S. flexibilis* thus had to deal with interractions between varieties of *Porites* at "Lone Tree", *Porites* and *Acropora* at "Loomis", whereas at the "Vicki's" site, the interactions were much more varied. Vicki's reef was an environment that was very, very competitive. We found at that site that the amount of flexibilide 8 varied significantly ie became noticeably higher relative to dihydroflexibilide 9 and sinulariolide 7. There is an implication here as flexibilide 8 is the allelopathic agent, ie our anti competitor agent. Our preliminary results suggest that there is indeed a greater variation in this component at the more competitive site relative to the sites where *Sinularia flexibilis* was competing with only one or two different species. I'm fairly confident that, as we get more data on the distribution of flexibilide 8 and sinulariolide 7 the full story of the variation between sites and whether in fact there is a correlation between species diversity and concentration of allelopathic components, will be revealed. Fig.4. shows that both sinulariolide 7 and flexibilide 8 have a common precursor, and may interconverted by enzymes, depending on the need for different amounts of each.

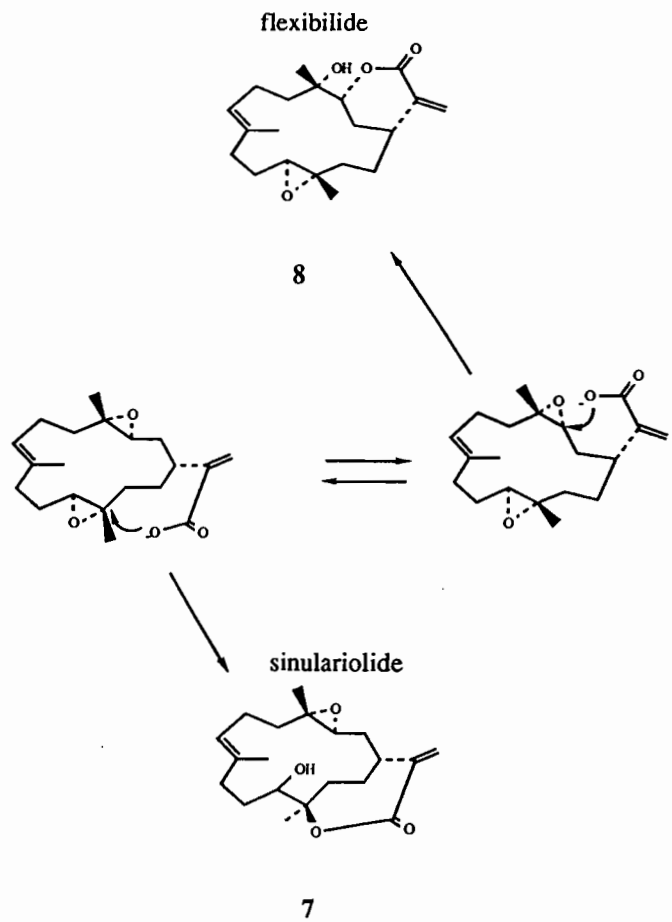


Fig. 4 : Possible enzymatic interconversion of sinulariolide and flexibilide

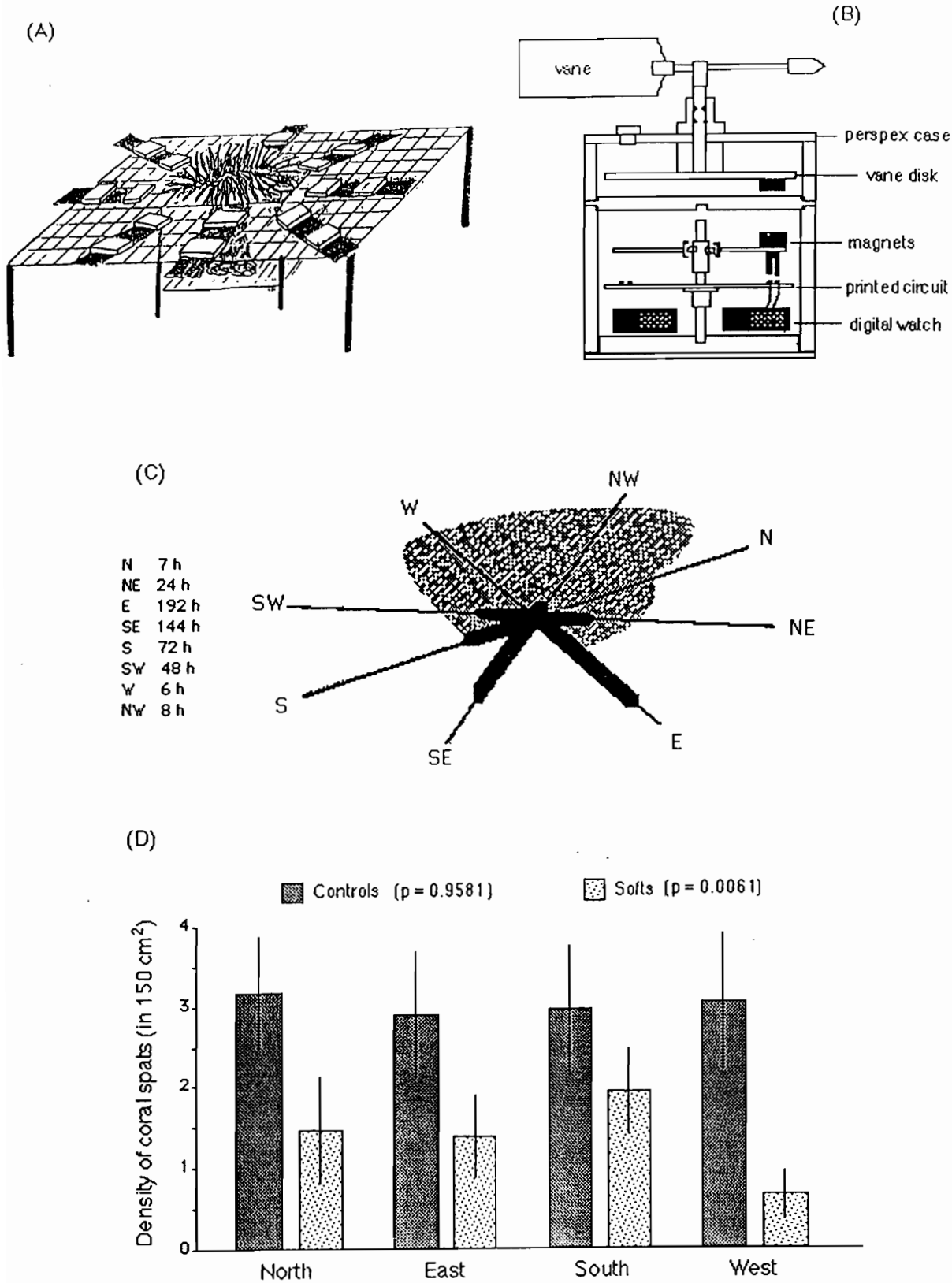


Fig. 5 : Directional aspects of allelopathy : *Sinularia flexibilis* on settlement of coral spat.

The final little story is another relatively new study involving experiments carried out by Mauro Maida. This involves the question of whether we can show the dependence of allelopathy on current directions. This involved setting up a series of experiments in which a soft coral was placed in the centre of a grid, and settlement plates are set at different distances from the soft coral radiating out along the eight directions of a compass (Fig.5a). The first experiment was set up using coral slabs attached to plastic sheets at Orpheus Island. What we found in this experiment was first of all that the settlement plates were not settled upon at all by coral spat. All settlement occurred on the underside of the plastic sheet, and we had to make measurements and assessments on the underside of the supporting structure rather than on the settlement plates.

How did we determine the flow of current in the study site ? Mauro designed and produced, with the help of AIMS technicians, a rather clever low cost little device for measuring the predominant direction of current flow in a local environment. This involved a series of stopwatches that were located within a circular drum (Fig 5b). Each stopwatch corresponded with the direction of one of the eight points of the compass, and all stopwatches were set to zero time at the start of experiment. Once they were activated they each started recording the time elapsed, unless the current vain was pointing in their direction. At that time a pair of contacts shorted out the watch corresponding to that current direction and caused it to stop. The other seven stopwatches continued to record, and in this way, we were able to assess the amount of time a current flowed in any one direction. We found that the direction of current flow was predominantly from the east ie towards the west (Fig. 5c). When you look at the difference between coral settlement on the underside the plastic sheet, you can see very clearly that the westerly side is significantly more inhibited relative to the other directions of the compass (Fig.5d) (13)

I believe that these studies have very interesting implications especially in areas of reef that have been devastated by cyclones, storms or after Crown of Thorns starfish plagues. After any natural disaster, the first settlement tends to be by soft corals. It is likely that *Sinularia flexibilis* and some *Sarcophyton* species inhibit the subsequent settlement of a large number of different hard corals, and in fact effect the distribution of hard corals that ultimately replenish a devastated reef. Hopefully we will have a lot more to say about these aspects in the next few years, as the results of our experiments come to fruition.

It only remains for me now to thank : the Organising Committee for the invitation to present this paper; my major collaborators in this work : Dr Bruce Bowden in the area of Chemistry; Dr Paul Sammarco in the area of ecology; and the many graduate students who have worked with us over the years. Our funding base was provided initially by Roche Products Australian, and then by Australian Research Grants Commission the Australian Research Council, Marine Science and Technology Grants Scheme and more recently by a number of pharmaceutical companies.

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JCC : ljb : 0817

Acknowledgements

Chemistry

Dr Bruce Bowden
Dr Tony Carroll
Dr Andrew Heaton
Dr Yasuto Uchio
Dr Dianne Tapiolas
Dr Gabi Konig
Rick Willis
Priscila Leone
Rocky de Nys
Jimmy Janairo

Biology

Dr Paul Sammarco
Dr Stephane La Barre
Dr Perry Alino
Dr Leith Webb

Tess Aceret
Mauro Maida
Mikel Becerro

Biological Collaborators
Dr Margaret Clayton (Monash)
Phil Alderslade (Darwin Museum)
Dr Russ Babcock (AIMS)
Dr Bette Willis (JCUNQ)
Dr Mike Capra (QUT)
Dr Mike Pass (U of Q)
Dr Dick Miller (Temple, USA)

Funding

ARGC, ARC
MSTGS, ARC
Roche, Pharmamar
James Cook University
Australian Institute of Marine Science

Troisième Symposium sur les substances naturelles d'intérêt biologique de la région Pacifique-Asie

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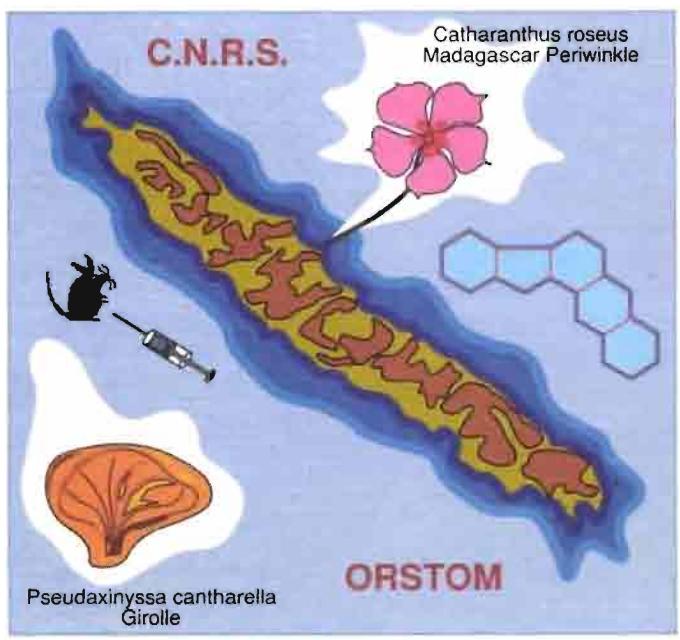

Editeurs : Cécile DEBITUS, Philippe AMADE,
Dominique LAURENT, Jean-Pierre COSSON