Evaluation of some methods for quantitatively assessing the toxicity of heavy metals to corals

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Abstract

The effects of dissolved copper on respiration, nutrient uptake and release, pigments, and zooxanthellae expulsion were investigated in Montipora verrucosa. The LC50 was determined to be 0.046 mg/L Cu (II). No significant change was detected in any other function after exposure to Cu (II) between 0.01 and 1.0 mg/L, except that polyps were visibly bleached and zooxanthellae expelled in proportion to copper concentration and to LC50. Bleaching in response to short-term exposure was slowly reversible in subdued light but was reinstated upon exposure to full sunlight in proportion to loss of the pigment S320. It is proposed that both zooxanthellae and S320 may hold an important place in the self-protection of corals against toxic chemicals.

Introduction

Heavy metals in the tropical marine environment have received little attention with respect to their potential toxic effect on reef-building corals (Mitchell and Chet, 1975; Evans, 1977; Brown and Belley, 1982). Evans (1977) found that continued exposure of Montipora verrucosa and Pocillopora damicornis to a dissolved copper concentration of 0.01 mg/L (as cupric sulphate) killed both species of coral within six days. This concentration was quoted as being slightly above ambient for coastal waters off Oahu, Hawaii (Evans, 1977).

In quantifying toxicity of metals to an organism, one would normally establish a 48 h or 96 h LC50, i.e., the concentrations of a chemical which kill 50% of the test individuals in 48 or 96 h. Herein lies an immediate difficulty in using this approach for corals, as the "individual" may not be easily identifiable or defined: the degree of coloniality which exists between polyps within a coral head will affect the decision as to what may be regarded as an individual (Hubbard, 1977; Shelton, 1978). This relationship may also vary between colonies. Additionally, determining the point of death of a polyp is not straightforward.

There are two approaches to assessing toxicity: 1) acute exposure to relatively high concentrations of toxicant, and 2) chronic exposure to low concentrations. While acute LC50 values are useful for comparing the relative toxicity of various agents to a species or between species, the assay has two major limitations. Firstly, LC50 values do not necessarily imply that 50% of the test individuals survive exposure at that concentration over time; it is possible to have 100% mortality of the organism some time later, even if they have been removed from exposure to the pollutant. Secondly, the acute LC50 values give no indication as to the lower limit of chronic exposure to a pollutant which would deleteriously affect the growth and reproduction of organisms constituting a population.

There is a need, therefore, to establish methods for quantitatively assessing both the acute and chronic toxic influence of pollutants on coral physiology, aspects of physiology which might be used are respiration, uptake and excretion of nutrients, loss of symbiotic algal pigment, and inhibition of reproduction and growth. The latter two parameters require long-term studies, and, as a more
rapid means of evaluating toxicity would be desirable, the objectives of the investigations described in this paper were: 1) to obtain a 96 h LC50 and 2) to evaluate the use of respirometry, nutrient uptake and excretion, and loss of zooxanthellar pigment as quantitative estimates of the toxic response of Montipora verrucosa to dissolved copper (II).

Materials and Methods

As time for conducting the investigation was limited, it was decided to confine the experimental corals to acute exposure to total copper. A water standard of pure cupric chloride, CuCl2·2 H2O was made up in deionized water, equivalent to 100 mg/L as copper (II) (1.57 mM Cu2+). Test solutions were prepared by appropriate dilution of the water standard with sand-filtered sea water; Cu background was not measured but was assumed to be close to that reported for other coastal waters (about 0.002 mg/L) (Burton, 1976). The proportion of cupric ion at each dilution also was not determined although unionized Cu(OH)2 and CuCO3 would be expected as significant Cu species (Dirzo and Yamamoto, 1972) at the lower Cu concentrations.

Corals

Montipora verrucosa branches were collected from the reef flat on Coconut Island, Kaneohe Bay, Oahu, Hawaii, from a depth of 2-3 m and transferred to holding tanks at the Hawaii Institute of Marine Biology. The corals were maintained in running sea water for 24 h prior to use in experiments and shaded from strong sunlight to prevent possible loss of zooxanthellae. All coral branches used in any given experiment were obtained from the same colony.

Respirometry

Dark respiration of corals was measured using a 151 Dissolved Oxygen Probe model 57 inserted in a closed volume of filtered sea water (600 ml). A schematic diagram of the experimental system is shown in fig. 1.

The respiration chamber was placed in a bath of running sea water to maintain constant temperature (about 26 °C). The experimental system was kept in a dark room supplied with red lighting to minimize photosynthetic activity of symbiotic algae. Corals were placed in the respiration chamber and allowed to recover from handling for 30 min. During this period, filtered sea water saturated externally with air was allowed to run through the respiration chamber. The water flow was then shut off and the dissolved oxygen concentration in the chamber monitored for a period of 60 min. The system was then flushed with aerated, filtered sea water for a further 30 min, and either (a) respiration of the coral was measured over 60 min. (control), or (b) copper (II) solution run through the system for one hour after which time the flow was shut off and the respiration measured over 60 min (tests). Background respiration of the system without corals was measured for both the sea water and copper solutions.

Nutrients

The same experimental system was used as for respirometry. Duplicate water samples were taken before and after incubation for 60 min in the chamber and immediately frozen for subsequent analysis. To test whether the presence of copper in the water samples affected the nutrient analyses, a separate experiment was conducted wherein corals were pre-exposed to copper for 60 min in a separate vessel before being transferred to the respirometry chamber. Water samples were then taken from the chamber at the beginning and end of a 60 min period, having first allowed 30 min acclimation. A total of three runs was carried out.

Phosphate, nitrate/nitrite, and ammonium were determined using a Technicon Autoanalyzer II. Two replicate analyses each of water sample were conducted.

Loss of Zooxanthellae

Quantification of loss of zooxanthellae was attempted using a chlorophyll extraction procedure. Montipora branch tips were exposed to copper (II) concentrations of 0.05, 0.05, 0.05, 0.05, 0.05 and 0.05 mg/L for 1 h. Controls were maintained in filtered sea water. After exposure, all corals were placed in clean sea water and kept under reduced light for 24 h. The corals were then placed in sea water to remove extruded zooxanthellae, ground with a mortar and pestle a
homogeneous paste, and an aliquot removed for chlorophyll analysis. Protein analyses were performed on the same aliquots after extraction of chlorophyll in order to standardize comparisons between coral branches in the form of micrograms chlorophyll-a/mg protein.

Chlorophyll extraction was carried out by adding 5 ml of acetone to the crushed coral sample in a test tube and holding in a refrigerator for 24 h. Each extract was mixed thoroughly before centrifuging and then decanting the supernatant. All samples awaiting processing were kept in the dark, on ice. The supernatants were allowed to warm to room temperature (approximately 30 min) before measuring absorbances at 630 nm and 687 nm (Jeffrey and Humphrey, 1973). Residues from centrifugation were frozen until ready for protein assay. Coral tissues were removed from the skeletal element by digestion in 1 M aqueous sodium hydroxide for 10 min at 50 °C followed by a rinse with deionized water until the skeletal fragments appeared clean. The sodium hydroxide extract was made up to a known volume and aliquots subjected to a modified Lowry protein assay as described by Eastman (1972).

A second series of solutions were prepared with copper concentrations of 1.0, 0.5, 0.1, 0.05, and 0.01 mg/l. Montipora branches were exposed to the copper solutions for 1 h and then kept in 600 ml glass jars containing filtered sea water for 24 h. After this period, zoanthellae which had been expelled by the corals in response to copper exposure were centrifuged from the sea water and examined under a Zeiss fluorescence microscope for pigment content. Samples of zoanthellae which were not expelled from the corals were also inspected after isolation from the crushed tissues. This was achieved by repeated agitation of the crushed coral with chilled sea water and filtering the washings through a 70 micron nylon mesh. Zoanthellae were centrifuged out, rinsed with filtered sea water, centrifuged again, resuspended in 1 ml of sea water, and an aliquot placed onto a microscope slide for inspection. Counts of fluorescent and nonfluorescent cells were made under both ultraviolet and white light illumination.

96 Hour LC50 Assay

Groups of ten small Montipora branch tips were exposed to copper (II) concentrations in sea water of: 0.10, 0.05, 0.03, 0.02 and 0.01 mg/l. Control corals were kept in filtered sea water. Each branch tip was held in a glass jar containing 100 ml of solution which was changed every 12 h. The corals were inspected regularly and their condition recorded. Corals were deemed dead when polyp tissue could no longer be seen within the calices. The corals were observed for a total period of 168 h.

Histology

Effects of copper at the cellular level were observed simultaneously with toxicity assays. Histological studies were made on corals which had been exposed for one hour to copper concentrations of 0.0, 1.0, 0.5, 0.05, and 0.01 mg/l and kept for 24 h in filtered sea water. A second series of corals was exposed to 1.0 mg/l of copper (II) for one hr before transfer to sea water. Coral samples were removed and fixed every hour thereafter for a period of 6 h in an attempt to observe the process of zoanthellae expulsion. Corals were fixed in 10% formalin in sea water for 24 h and decalcified with 2% formic acid with several changes over 6 h. Decalcified tissues were processed to wax using an Autotechnicon model 20 automatic tissue processor. Embedded tissues were sectioned at 6 microns using an American Optical model 820 microscope and stained with hematoxylin and eosin for inspection under the light microscope.

Recovery from Exposure to Copper

Branch tips of Montipora were exposed to copper concentrations of 1.0, 0.5, 0.1, 0.05 and 0.01 mg/l for 1 h, placed into tanks provided with running sea water, and inspected regularly to record any recovery which occurred following bleaching.

Small heads of Montipora (approximately 10 cm diameter), exposed to copper concentrations of 0.1, 0.05 and 0.01 mg/l for 1 h, were transplanted onto the reef at a depth of 1-2 m, to compare recovery in holding tanks with that in the field.
Results

Respirometry

Dissolved oxygen readings were found to fluctuate during the first 10 min after closing off the water supply to the respirometry chamber. The reasons for this are unclear but may be related to pressure changes as a result of closing the valves or a response to reduced water flow through the system affecting diffusion rates across the electrode zebrines. For this reason, oxygen consumption rates across the electrode zebrines which occurred between 20 and 40 min were taken as the drop in dissolved oxygen which occurred between 20 and 40 min. It can be seen that there was no consistent effect of copper (II) on respiration rate; there was a tendency for oxygen uptake to decrease after exposure to copper (II), although it actually increased in several samples. Measurements on corals without exposure to copper (II) also showed slight decreases in oxygen uptake. Overall, the mean rate of oxygen uptake for all corals before copper (II) exposure was not significantly different from that after copper exposure. Measurements on corals after a period of 24 h also failed to show any consistent effect of exposure even to 1 mg/l Cu II on respiration, although at this point the coral already was bleached. However, respiration in all cases was significantly greater than in seawater alone.

Table 1. Montipora teretea: Decrease in oxygen concentration (mg/l/40 min) before and after exposure to copper (II) (1 mg/l).

<table>
<thead>
<tr>
<th>Before exposure to copper (II)</th>
<th>After exposure to copper (II)</th>
<th>Background respiration (no coral)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.20</td>
<td>-0.15</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.30</td>
<td>-0.15</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.40</td>
<td>-0.20</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.45</td>
<td>-0.35</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.50</td>
<td>-0.40</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.55</td>
<td>-0.45</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.60</td>
<td>-0.50</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.65</td>
<td>-0.55</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.70</td>
<td>-0.60</td>
<td>-0.20</td>
</tr>
<tr>
<td>-0.75</td>
<td>-0.65</td>
<td>-0.20</td>
</tr>
<tr>
<td>Mean</td>
<td>-0.32**</td>
<td>-0.26</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.138</td>
<td>0.131</td>
</tr>
</tbody>
</table>

**Control, not exposed to copper**

**Variance ratio of means before/after: t=1.11 (d.f. 5, 9), not significant:**

Nutrients

Changes in nutrient concentration of sea water containing Montipora branch tips before and after exposure to 1.0 mg/l of copper (II) are given in Table 2. There was no consistent effect of copper (II) on any of the nutrients monitored. Differences between “before” and “after” exposure varied from +0.12 to -0.09 micromole/l for nitrate plus nitrite, 0.07 to -0.12 micromole/l phosphate, and +0.12 to -0.04 micromole/l ammonium. Thus each of the nutrients was taken up in some experiments and excreted in others, with each acting independently of the other two. Control corals exhibited a short term variation in nutrient uptake of a magnitude similar to that observed in copper-exposed corals.

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Table 2. T. pyriformis: Net change in dissolved nutrient concentration during incubation (mole/l)*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phosphate</th>
<th>Nitrate/Nitrite</th>
<th>Ammonium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B-A</td>
<td>B</td>
</tr>
<tr>
<td>Background (no corel)</td>
<td>-0.01</td>
<td>0</td>
<td>-0.01</td>
</tr>
<tr>
<td>Controls (no copper)</td>
<td>0</td>
<td>-0.01</td>
<td>-0.03</td>
</tr>
<tr>
<td>Test (copper exposed)</td>
<td>+0.02</td>
<td>0</td>
<td>+0.02</td>
</tr>
<tr>
<td></td>
<td>+0.12</td>
<td>-0.12</td>
<td>-0.12</td>
</tr>
</tbody>
</table>

*Mean of 4 replicates.
**A=Before exposure to copper, B=After exposure to copper.

Loss of Zooxanthellae

Table 3 summarizes the results of chlorophyll assays. Visually, there was a gradation of bleaching proportional to the concentration of copper (II); higher concentrations of copper elicited a greater degree of bleaching. This trend was not supported by chlorophyll per unit protein assays, where an analysis of variance indicated no significant variation between treatments (Table 4).

Table 3. A. pyriformis: Mean chlorophyll content of branch tips exposed to a range of copper concentrations (g chlorophyll/mg protein).

<table>
<thead>
<tr>
<th>Copper (mg/L)</th>
<th>Mean</th>
<th>S.D.</th>
<th>n</th>
<th>Coefficient of Variation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>4.35</td>
<td>0.97</td>
<td>6</td>
<td>22.3</td>
</tr>
<tr>
<td>0.01</td>
<td>3.35</td>
<td>0.52</td>
<td>5</td>
<td>15.5</td>
</tr>
<tr>
<td>0.02</td>
<td>2.27</td>
<td>0.55</td>
<td>5</td>
<td>16.8</td>
</tr>
<tr>
<td>0.03</td>
<td>1.80</td>
<td>1.20</td>
<td>5</td>
<td>31.3</td>
</tr>
<tr>
<td>0.04</td>
<td>6.32</td>
<td>0.52</td>
<td>5</td>
<td>12.9</td>
</tr>
<tr>
<td>0.05</td>
<td>3.37</td>
<td>0.53</td>
<td>5</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Table 4. A. pyriformis: Analysis of variance of mean chlorophyll content of branch tips exposed to a range of copper (II) concentrations.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between treatments</td>
<td>5</td>
<td>6.00</td>
<td>1.20</td>
</tr>
<tr>
<td>Within treatments</td>
<td>22</td>
<td>11.70</td>
<td>0.50</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>17.50</td>
<td></td>
</tr>
</tbody>
</table>

F=2.40 (D.F. 5,22)

P=0.05 (D.F. 5,22), i.e., there is no significant difference in variance between treatments.
A further chlorophyll assay was carried out on corals exposed to 1.0 mg/L of copper (II) for 1 h followed by 24 h in sea water. The results (Table 6) indicate a significant difference (p<0.01) in mean chlorophyll content between test and control corals.

Table 6 gives the results of microscopical inspection of expelled zooxanthellae. Corals from 1.0 and 0.5 mg/L Cu II exposure appeared equally bleached, at concentrations of 0.1 mg/L Cu II and less, bleaching visually appeared proportional to copper concentration. Controls retained their normal color. Table 6 indicates that the number of zooxanthellae expelled appeared to be proportional to copper (II) concentration up to 0.3 mg/L Cu II. The number of zooxanthellae expelled at 1.0 mg/L Cu II did not appear to the same extent. Corals in control and 0.01 mg/L Cu II did not appear to expel zooxanthellae. The proportion of expelled algal cells which had lost their pigment was also proportional to copper concentration. Zooxanthellae remaining in the coral tissues after exposure to copper (II) appeared healthy in all test concentrations of the metal. Thus, zooxanthellae expelled from Montipora showed a degree of pigment loss proportional to exposure concentration. No attempt was made to quantify the number of zooxanthellae expelled as a proportion of the total number present in the coral tissues.

Table 5. Montipora: mean chlorophyll content of branch tips exposed to 1.0 mg/L copper (II) (mg chlorophyll/mg protein).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.50</td>
<td>0.66</td>
<td>14.2</td>
</tr>
<tr>
<td>Test</td>
<td>2.50</td>
<td>0.30</td>
<td>11.5</td>
</tr>
</tbody>
</table>

\[ t = 3.56 \] d.f. 4 (p<0.001), therefore variances not significantly different.

\[ t = 3.68 \] d.f. 6 (p<0.001), therefore the means are significantly different (p<0.01)

Table 5. Montipora: Proportions of normal and bleached zooxanthellae expelled from branch tips after exposure to a range of copper (II) concentrations.

<table>
<thead>
<tr>
<th>Copper Conc. (mg/L)</th>
<th>Total Cells Counted</th>
<th>Total Normal</th>
<th>% Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>810</td>
<td>76</td>
<td>2.3</td>
</tr>
<tr>
<td>0.50</td>
<td>969</td>
<td>74</td>
<td>14.2</td>
</tr>
<tr>
<td>0.10</td>
<td>516</td>
<td>76</td>
<td>56.7</td>
</tr>
<tr>
<td>0.05</td>
<td>134</td>
<td>17</td>
<td>(100.0)</td>
</tr>
<tr>
<td>0.01</td>
<td>4</td>
<td>1</td>
<td>(100.0)</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Fifty percent mortality figures were obtained for *Montipora* branch tips at copper concentrations of 0.0, 0.10, 0.05, and 0.01 mg/l. Even after 16h of exposure, e. g., only 1 out of 10 *Montipora* branch tips was dead in 0.02 mg/l Cu II and none were dead at 0.01 mg/l. All corals were bleached by these concentrations and exhibited variable degrees of skeletal exposure, while controls appeared normal. Fig. 2 plots the time taken for 50% of the branch tips to die at each experimental copper (II) concentration and yields a 96h LC50 of 0.048 mg/l.

![Graph showing time for 50% mortality vs copper concentration.](image)

**Fig. 2.** Time for 50% mortality of *Montipora* branching at various copper concentrations.

**Histology**

Inspection of histological sections of *Montipora* following exposure to copper (II) concentrations of 1.0 and 0.5 mg/l confirmed extensive zooxanthellae loss. Additionally, columnar ectodermal tissues showed extensive disruption at 1.0 mg/l but not at 0.5 mg/l Cu II. At concentrations lower than 0.5 mg/l, no obvious histological differences from control tissues could be discerned.

The mechanics of expulsion of zooxanthellae was not easily evaluated, as interpretation of the histological sections was difficult. However, zooxanthellae
were observed to lie outside endodermal tissues within the enteron of corals exposed to 1.0 and 0.5 mg/L Cu II but not so in controls nor in corals exposed to concentrations lower than 0.5 mg/L. It is assumed that the zooxanthellae were extruded via the pharynx, although this was not actually observed in the sections. Extruded zooxanthellae were invariably found in thick succ in all experiments.

Recovery from Exposure to Copper (II).

Laboratory Study

All test corals showed bleaching proportional to copper (II) concentration within 24 h following the exposure. At concentrations of 1.0 and 0.5 mg/L, large areas of skeleton were exposed due to tissue withdrawal, and there appeared to be some tissue degeneration. After 24 h, extensive diatom growth was observed on areas of exposed skeleton. Polyps were fully retracted but responded to mechanical stimulation and retained some zooxanthellae, especially in the tentacles. Diatom growth was reduced after 77 h, and polyps responded to mechanical stimulation. Between 77 and 197 h (8 d) after exposure, there was no further change in the corals. Change in condition thereafter was slow but gradually improved until by the 22nd d after exposure, although the corals were still bleached at all concentrations of copper, tissue had again covered the skeleton. By the 35th day after exposure, corals at 1.0, 0.5, and 0.1 mg/L Cu II were slightly pale in color compared with controls, although zooxanthellae were distributed throughout the tissues. Corals from 0.05 and 0.01 mg/L Cu II appeared similar to controls. On the 40th day after exposure, all treated corals appeared similar to controls.

During the period of recovery, corals were kept under subdued light. After regaining normal coloration, the corals were placed in full ambient sunlight. Within 24 h, corals had bleached in proportion to the initial copper concentration exposures. Control corals did not bleach.

Field Study

Coral exposed to Cu II for 1 h in the laboratory and then returned to the reef flat showed an early response similar to that of corals maintained in tanks. Montipora branches were 0.10 and 0.05 mg/L Cu II exposure exhibited bleaching, whereas those from 0.01 mg/L showed no difference from controls. However, during the second week after exposure, control corals also developed extensive bleaching on their upper surface coinciding with very low tide, and after 45 days, no apparent recovery had occurred in either test or control corals.

Discussion

The 96 h LC50 of 0.048 mg/L for total Cu is in the range of that reported for many other aquatic species [Black et al., 1976], although no similar data are available for other corals with which it may be compared. This copper concentration is approximately 50 times greater than that of coastal sea water (Burton, 1976) but within the range of concentrations found in highly polluted inshore waters (Safran et al., 1969). The LC50 data also coincided with the observed bleaching and the expulsion of zooxanthellae. Because of this, the outcome of the LC50 experiment must be viewed with concern, as it may be inferred from the other experiments that lower concentrations of copper (II) over a prolonged period of time would have a detrimental impact on coral communities.

The apparent lack of effect of 1.0 mg/L of copper (II) (which may be considered a high concentration) on respiration, in unusual. Copper (II) is well documented as affecting respiration in a number of organisms (Scott and Major, 1972), and respiratory impairment is a primary manifestation of the toxicity of this element. That this was not observed in Montipora suggests that initially, the Cu II may be sequestered by zooxanthellae before the coral itself is affected to any large degree. At low concentrations of copper, relatively few zooxanthellae were lost, but polyps remained withdrawn for several days. Higher concentrations of copper resulted in coral tissue degeneration (initially confined to surface epithelia but ultimately resulting in massive tissue loss and polyp death), and zooxanthellae which were ejected showed varying degrees of pigment loss according to exposure concentration. The effect of copper (II) on the zooxanthellae seem likely to have occurred in the endodermal tissues, but as no
bleached zooxanthellae were observed in situ in histological sections, pigment loss may have occurred after ejection as a defensive detoxification mechanism of the polyp.

Experimenter was hampered by great variability in the data. Hulsemeyer and Randle (1975), in their review of methods for assessing coral growth, commented on the variability of data in that "even when systematic variation is allowed for, a substantial amount of 'noise' remains." Highly variable data were also reported by Barnes and Crossland (1982), where coefficients of variation for calcification in Acropora acrorhiza ranged from 20 to 30% and occurred over time, within and between coral colonies, and within any group of experimental replicates. Similar coefficients of variation in calcification rates and protein assays can be found in data published by Kendall et al. (1982) on the effects of drilling muds on growth and metabolic state of Acropora cervicornis.

This latter study made some relevant observations concerning the use of protein assay as a basis for normalization of experimental results. It was found that protein varied significantly over a 24 h period and, furthermore, controls were very different from each other on different days, depending upon the mode of normalization chosen (protein, polyp number, skeletal weight). This may explain why protein-based normalization of the data presented in Table 3 failed to yield statistical support to visual observations in Porites exposed to a range of copper concentrations; reflectance colorimetry may prove to be a more useful technique for quantifying the bleaching. The graded response also could be due to varying degrees of tissue withdrawal in response to the copper "bleaching." This could be an artifact induced by increased transparency of the tissues as they retracted into the fenestrated skeletal matrix, allowing the skeleton to become more visible. Histological inspection of coral exposed corals support this alternative, inasmuch as zooxanthellae were abundant in the endoderm of corals exposed to 0.05 mg/L Cu II and less. The data presented in Table 6, however, also suggest that few zooxanthellae are expelled from the coral at copper (II) concentrations less than 0.05 mg/L. As protein assays may be influenced by loss of zooxanthellae from the corals, the problems associated with protein as a basis for data normalization are compounded: the evaluation of respiration rates and nutrient uptake must also be normalized in order to make comparisons between experimental groups.

Data variability may be a major obstacle in attempting to establish a truly quantitative sublethal bioassay for coral polyps: any attempt to develop sensitive, quantitative estimates of the sublethal toxicity of metals (or any other chemical) to corals, must deal with the inherent "noise". Evans (personal communication) has suggested establishment of a large data base on natural variation in a range of physiological parameters in corals against which the effect of specific materials could be measured, but financially justifying the large time commitment required to establish it may prove difficult. The use of short-term procedures in addition to those described in this paper, e.g., copper growth rates and 14C photosynthetic activity of zooxanthellae, probably would also involve the use of unrealistically high concentrations of metal to be able to statistically separate metal effects from background noise. If it proved impossible to develop satisfactory short-term sublethal bioassays for coral intoxification, then the immediate alternative is for the more restricted long-term monitoring of low level exposures of the type described by Evans (1977).

Despite the limiting influence of noisy data in quantifying the effect of dissolved copper on Porites, the observed sensitivity of this coral to very short exposures at low concentrations is remarkable. Branch tips exposed for as little as 1 h to 0.03 mg/L of copper (II) showed initial signs of bleaching within 3 to 4 h after return to clear seawater and were extensively bleached after 24 h. Polyps typically were withdrawn after exposure and remained closed for several days. In the absence of major respiratory or nutrient effect, the action of Copper (II) remains unknown but would appear to be prolonged. This copper appears to influence both the coral and zooxanthellae, but attempts to apportion the contribution by either the overall toxic response is difficult. The results suggest that there is input from the coral and zooxanthellae on an individual basis and that the proportion may depend upon the particular exposure concentration.

The process of expulsion could be stimulated by metabolites from degenerating zooxanthellae and may also initiate coral tissue death. The pathway of expulsion appeared to agree in general with observations of Tang and Nicholls (1931) insofar as zooxanthellae seemed to be ejected by way of the enteron and pharynx.

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One major difference, however, was that zooxanthellae were discharged directly into the enton from the endoderm, and no evidence was found to suggest transport of zooxanthellae in amoebocytes to mesenterial filaments as described by Yone and Nicholls. These authors found the mechanism of expulsion to be consistent when elicited by increased temperature, prolonged periods in the dark, starvation or oxygen deprivation. The action of copper on Montipora zooxanthellae thus appears to be different from what may be considered as normal environmental perturbations.

The long-term bleaching effect is probably due to the length of time required to regenerate zooxanthellae. The period of five weeks for complete regeneration in Montipora compared with six weeks in Actinora palmae and Montastrea annularis (Carper, 1978) and eight to twelve weeks in Porolithon durhamensis, Porolithon bonedae and Porites lobata (Cayre and DiSalvo, 1982). An interesting observation in the regeneration of zooxanthellae was the apparent sensitization of the coral to ambient light after recovery under reduced light intensities; the amount of bleaching coincided with the proportion of zooxanthellae which had been regenerated during the five-week recovery period. A possible cause is that the regenerated zooxanthellae were not light-adapted and lacked the ultraviolet-absorbing pigment "S320" (Zielinski and Tuck, 1981), subsequent exposure to full ambient light caused the UV destruction of these zooxanthellae.

Measurements of S320 in corals after copper-induced loss of zooxanthellae showed that they contained less S320 than controls (Table 7). The ratio of chlorophyll-a to S320 suggested that the S320 was associated with the zooxanthellae. Furthermore, incubation of S320 extract with copper (II) did not result in a decrease, which suggests that the action of the copper is on the zooxanthellae and not on the S320. This would support the contention that reduction of S320 in copper-exposed corals is associated with zooxanthellae expulsion and could be visualized by measuring S320 in corals before and after recovery of zooxanthellae under both reduced and full light conditions. In the field, a major part of pollution damage to corals may be due to reduced self-protection from UV radiation.

Table 7. Montipora verrucosa: Changes in S320 (absorbance/nm diameter core) and chlorophyll-a (μg/75mm diameter core) following exposure to 1.0 mg/l copper (II).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>Ratio of S320 to Chlorophyll-a</th>
</tr>
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<tbody>
<tr>
<td>S320 Test</td>
<td>0.107</td>
<td>0.013</td>
<td>0.104</td>
</tr>
<tr>
<td>S320 Control</td>
<td>0.161</td>
<td>0.011</td>
<td>0.093</td>
</tr>
<tr>
<td>Chl-a Test</td>
<td>1.033</td>
<td>0.120</td>
<td>1.000</td>
</tr>
<tr>
<td>Chl-a Control</td>
<td>1.729</td>
<td>0.300</td>
<td>1.000</td>
</tr>
</tbody>
</table>

The longer period required for corals to recover in the field when compared with laboratory studies suggests a synergistic effect of environmental variables on the response of Montipora to copper (II). In view of the sensitivity of Montipora to very short exposures to low concentrations of copper (II), synergism with other pollutants becomes important. Synergistic effects were highlighted by Antonius (1981) when describing the alarming "shut-down reaction" of stressed corals; rapid death of corals can result from exposure to a relatively harmless additional impact which would not normally damage a healthy coral. Synergistic interactions may also underlie the recent mass coral mortalities reported on the Pacific coast of Panama (Slynn, 1983).

Acknowledgements

We would like to thank the Pauley Foundation (USA) and the Company of Biologists (England) for generous financial support without which this work would not have been possible. We also wish to thank the scientists and staff of the Hawaii Institute of Marine Biology for guidance, help, and friendship extended throughout the Summer Program.
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Short term copper bioassay on the planula of the reef coral *Pocillopora damicornis*.

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Abstract

A short term (96 h) copper bioassay was done on the planulae of the reef coral *Pocillopora damicornis* and the 24 h, 48 h and 96 h median lethal concentrations (LC50) were determined graphically and by formal probit analysis. Concentrations of copper (added as CuCl2) ranged from 10-1000 μg l⁻¹ (ppb). A toxicity curve was plotted as the experiment proceeded. Planulae under stress showed signs of contraction, mucous secretion, zoanthellae-expulsion and breakdown of the body wall. Comparisons with previous studies show the planula of *P. damicornis* to be the more resistant life stage to heavy metal toxicity, surviving in higher concentrations for longer periods of time than the adult coral colony. In spite of the difficulty in determining death in colonial organisms, the adult coral colony of *P. damicornis* is recommended for future use in toxicity studies being the more sensitive stage of the coral life cycle.

Introduction

Research into the effects of heavy metal pollution in coral reef systems has increased in recent years (Bryan, 1980 and Howard and Brown, in press). Studies of heavy metal toxicity on hermatypic corals, however, have been limited to qualitative laboratory observations made at various exposure times across a range of concentrations.

The relative sensitivity of larvae and adults of the same species to the heavy metal copper has been investigated in several invertebrate. These include: barnacles (Prylianch and Nott, 1948), sponges, tubeworms, molluscs and brine shrimp (Wiseley and Blich, 1967), crabs and lobsters (Conner, 1972), and bivalves (Cabrera et al., 1973, 1974, 1977). In the case of hermatypic corals, however, research has been confined to all instances to the adult stage in the life cycle (Livingston and Thompson, 1971; Sreekantharam and Sogata, 1972; Mitchell and Chet, 1975; Evans, 1977).

The purpose of this study was to establish the range of copper concentrations in seawater that induce lethal and sublethal effects on the planulae larvae of *Pocillopora damicornis*, and to arrive at an approximation of the 24 h, 48 h and 96 h median lethal concentrations (LC50).

*P. damicornis* is a cosmopolitan Indo-Pacific reef coral. The planulae larvae of *P. damicornis* are 1.5 mm in average length and highly contractile, varying in shape from a sphere to an ellipsoid with the aboral end broader and rounder than the oral end (Edmondson, 1986). They are non-feeding or lecithotrophic non-calculating, solitary, planktonic organisms (Marizigua, 1972). The planula is covered by uniform short cilia which function directly after release from adult. Marizigua (1972) observed and classified the movements of planulae into 7 classes. For the purpose of this study, these classes have been regrouped into three basic types of behavior. These are: 1. change of body shape, 2. stationary rotation about an oral-aboral axis and 3. active swimming. The total immobilization of the planula can be considered an indication of death (Sand et al., 1976), allowing the measurement and calculation of the LC50.

Materials and Methods

A colony of *P. damicornis* (approximately 10 cm diameter) was collected and placed in a 31 container maintained at a flow of 0.5 to 1.0 l min⁻¹ of seawater. Water flowing through the containers was channeled into collectors made from plastic beaker bases with walls constructed from 100 micron plankton netting. Collection of newly released larvae was done on 26 June 1982. Release of planulae has been stimulated by warming the water to 35°C for a few minutes (Edmondson,
1946). However, this was not done as this method tends to produce damaged and immature specimens. Three hundred "actively swimming" planulae (i.e., exhibiting all the three modes of movement previously described) were separated and put in Millipore-filtered (0.45 micron) natural seawater for a 48 h acclimation period. Throughout the acclimation period, temperature of the water was kept constant at 27°C. The seawater was aerated continuously but not changed. Mortality was observed at the end of the acclimation period, prior to the start of the bioassay. It has been suggested that organisms should not be used if at least 15% mortality is exhibited during the 48 h period immediately before the beginning of a bioassay (Rand et al., 1975). This was not observed.

Round, widemouth, clear-glassed Jars (472 ml volume) were used in the bioassay. All containers and glassware were washed twice with detergent, rinsed twice with 50% nitric acid, distilled water and finally with Millipore-filtered seawater. All concentrations of copper were made using CuCl. The copper ion has been recognized as the toxic form of copper in seawater (Steensen-Nielsen and Wies-Anderson, 1970). A 100 mg l⁻¹ stock solution in distilled water was made, from which measured quantities were taken and diluted in Millipore-filtered seawater for the required concentrations of the bioassay. It must be noted that the concentrations in the study were that of added copper. The initial concentration of copper in the water used to make the solutions was not determined.

The selection of test concentrations was made based on the progressive bisection of intervals on a logarithmic scale suggested by Rand et al. (1975). Use of log concentrations where each is 55 to 57% of the next highest concentration assures reasonable accuracy when plotting the toxicity curve.

Ten actively swimming planulae were then placed in each jar previously filled with 100 ml of the test solution (resultant depth of the solution in the jar was 2.2 cm). Two replicates were made for each concentration, including two controls which were Millipore-filtered natural seawater. The bioassay was conducted in a shallow holding tank in the shade of an awning. The larvae received indirect sunlight and the temperature of the solutions varied from 26.5°C to 27.0°C, being kept relatively constant by submerging the jars halfway in the holding tank which had natural seawater continuously flowing through it. The jars were aerated constantly throughout the 96 h bioassay period by a stream of air from a glass tube. Only glass ware was in contact with the test solutions and planulae. This was a short-term static bioassay. It was not necessary to renew test solutions during the duration of the study. The bottles were covered with parafilm to insure cleanliness.

At the designated time intervals, the planulae were observed. Any planulae which remained completely immobile even after gentle prodding with a glass pipette, were recorded as deaths; they were removed and put in fresh Millipore-filtered natural seawater and continuously observed for signs of recovery (i.e., resumption of any form of movement).

The data were analyzed in the manner described by Rand et al. (1975). The median lethal concentrations were determined graphically and through probit analysis and were then plotted on the toxicity curve. The probit analyses function of the computer language SAS (Statistical Analysis System) was used to obtain the 95% confidence limits and the slopes of the individual determinations of lethal concentrations for different time lengths. The chi-square analysis of the same system was used to test for homogeneity.

Results

The chi-square analysis of the data sets done at each time interval revealed homogeneity of the organisms (p0.05). This was expected as all the test organisms were collected from a single planulizing colony of D. linedata.

Planulae were observed against a bright background. Prior to the start of the bioassay, they were oblong in shape and exhibited dark brown lines of zooidal antecellae in their intercellular regions. They were swimming actively, chomping in shape at the moment of contact and returning to their original tubular shape after several seconds. The planulae were observed to contract into spherical shapes and remain contracted, however, upon exposure to the copper solutions. This was not observed in the control jars.

As the bioassay proceeded, the planulae in the higher concentrations (i.e., >1000 ppm added copper) extruded "filaments" and "noodles," similar to those ob-
served by Sinkевич and Loya (1979) in planulae of the Red Sea coral Siderastrea shelfae. The filaments were curled into ring-like shapes and were found mostly near the oral end. The globular nodules containing dark zoanthellae were found along the body wall of the planula in areas near both the oral and aboral poles. The planula appeared bleached in the later stages (after 5-6 h into the bioassay). Nucleus was also observed in the surrounding copper solution. Sinkевич and Loya (1979) have also observed such mucus-secretion and zoanthellar structures in planulae of P. damicornis. Rotational movement about an oral-aboral axis was still observed at this point. The planulae, however, were highly contracted into flattened, shortened forms. Contraction of this kind has been noted by Edmunds (1986) in planulae of P. damicornis exposed to fresh water.

In the later stages of the bioassay, the planulae showed destruction of the body wall. The epithelial layers at the oral end appeared torn. The nodules eventually became disconnected from the body wall but with parts of the wall still attached to them. This was followed by the lysis of the entire planula, i.e., disintegration of the body wall and loss of body form. The planulae now had totally irregular shapes, appearing as masses of mucus and tissue at the bottom of the jars. At this point there was cessation of all modes of movement.

The planulae in the lower concentrations, (i.e., <100 ppb added copper) did not exhibit this destruction of body form. The first effect observed was contraction into spherical shapes, then into more shortened, flattened forms. When prodded, the planula usually contracted more and/or began to rotate on their oral-aboral axis. They appeared “bleached” towards the end of the bioassay and minute amounts of mucus were evident in the surrounding solution. Nodules and filaments were also noted, mostly near the oral end. The nodules did not become detached, however, at any time during the bioassay.

Upon cessation of all modes of movement (even after gentle prodding) the planulae were removed from the copper solutions and placed in natural Villepore-filtered seawater. None of the planulae were observed to revive, (i.e., no resumption of movement was evident). All of them eventually exhibited sloughing off of the epidermal layer, and disintegration of body form after 24 h.

All the planulae in the control jars retained their ciliated or responded to prodding by changing shape or by moving. All three modes of movement were exhibited throughout the duration of the experiment. Bleaching was not observed in the controls but minute amounts of mucus were evident in the surrounding solutions. None of the controls settled at the bottom of the jars. The planulae were usually observed at the surface to a depth of about 1 cm. Nodules and filaments were not observed at any time.

The data and median lethal concentrations are shown in Table 1. The graphical estimates for the 24 h, 48 h and 96 h LC50's are shown in Fig. 1. To construct percentage mortality, per cent mortality was plotted on the vertical axis versus concentration on the horizontal axis. Mortality is on a probability or probit scale and concentration on a logarithmic scale. Because the probit scale never reaches 0 or 100%, such points were plotted with an arrow indicating their true position. Only one successive 0% and one 100% mortality value was used in each calculation, these being the ones nearest the center of range of concentrations.

The line was fitted to the points by eye, giving most consideration to points between 1% and 99% mortality (Rand et al., 1975). The graphical estimates are very close to those obtained by formal probit analysis with the computer.

The toxicity curve (Fig. 2) showing the progress of the bioassay was plotted using LC50's determined previously. The curve closely approaches an asymptote with time, but may not quite have reached it. Therefore, no threshold LC50's were determined in the present study. The 95% confidence limits of each LC50 is reported in Table 1 and is indicated as horizontal bars on the toxicity curve (Fig. 2).

Discussion

All previous work on copper toxicity has been confined to the adult stage of the coral animal. It is now possible to compare the effects of the metal at two different life stages. Evans (1977) exposed adult colonies of P. damicornis to solutions of copper sulfate at concentrations of 10 to 0.01 mg l⁻¹ (ppm).
Table 1. Experimental data. Estimates of median lethal concentration (LC-50) values using graphical and probit analysis at 27°C ± 0.4°C in natural millipore-filtered seawater using chloride solutions.

<table>
<thead>
<tr>
<th>Concentration of Added Cu (ppb)</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
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</table>

LC-50 graph 556 440 290 190 120 115 93 70 63
LC-50 probit 552 438 286 195 123 114 67 69 57
95% Conf. limits 740.21 850.84 375.33 252.56 164.88 148.58 120.03 121.39 96.32
Slope-probit 0.004 0.008 0.006 0.010 0.016 0.020 0.018 0.016 0.025

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Fig. 1. Median lethal concentration (LC50) as determined by graphical analysis.
Legend: 24 hour LC50 = 115 ppb is represented by circles and dashed line; 48 hour LC50 = 90 ppb is represented by triangles and dotted line; 96 hour LC50 = 63 ppb is represented by squares and solid line.
Fig. 2. Graph of the toxicity curve drawn as the experiment proceeded, from LC-50's determined graphically. Curve has become almost asymptotic with the time axis. The 95% confidence limits determined by formal probit analysis are shown for each LC50.
exposures of 0.1 mg 1-1 Cu (100 ppb) or greater, all coral colonies died within 24 h. Planulae used in the present study showed 50% to 90% survival at 24 h exposures of 100 and 180 ppb added Cu. Evans also noted that the coral colonies showed symptoms of "stress" with polype withdraw and whitened after 6 h exposure to 0.01 mg 1-1 Cu (10 ppb). All the planulae in the present bioassay were alive and showing no evidence of stress, i.e., the planulae were moving in the three modes of movement previously mentioned and retained their dark brown healthy coloration even after 48 h exposure to 10, 18, and 32 ppb added Cu.

Edmondson (1966) observed the effects of altered salinity and thermal conditions in the planulae of P. damicornis and suggested that the planula may be the more resistant life stage of the coral animal. The results of this study support that theory. The planula of P. damicornis appear to be more resistant to heavy metal toxicity than the adult, surviving in higher concentrations for longer periods of time. The causes of these differences in resistance between stages are not clear at the present time. There are marked differences in morphology and physiology between the various stages in the life history of the coral animal.

Various means by which copper is assimilated into the adult coral animal have been proposed by Howard and Brown (in press). In the planula, the copper may be incorporated as a soluble metal in food material; it may be directly incorporated by the zoanthenllae; and it may be associated with osmoregulatory or respiratory functions. As the planula is considered a lecithotrophic planktonic organism (Marzigan, 1972), the feeding activities of the planulae (if any) may not represent a significant input of metals. The possible incorporation of heavy metals by symbiotic zoanthenllae and their potential toxic effects on the host coral in terms of possible growth inhibition is discussed in detail by Howard et al. (in press). In both of these pathways, the heavy metals penetrate the tissues where they act intracellularly on metabolic processes. Heavy metal ions might not penetrate the tissues. They could exert their effect by absorbing onto the surface membrane of the organism. This could interfere with such vital processes as respiratory exchange or osmoregulation. Any of these mechanisms could be possible explanations for the contraction, bleaching, mucous-secretion by and lysis of planulae observed in the present study.

In any case, it is evident that further research is necessary in order to ascertain the exact manner by which the copper affects the coral to be able to fully interpret the observations in this paper. In spite of the difficulty in determining death in colonial organisms, the adult colony of Porites porites damicornis, being the more sensitive life stages, (i.e., less resistant to heavy metals), appears to have a greater potential as a test organism for research in toxicity experiments, pollution control and water quality determinations.

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Preliminary investigations into the occurrence and toxicity of commercial herbicide formulations in reef building corals

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Abstract

Pesticides were detected in several species of reef-building corals inhabiting the Pacific coastal waters of Panama, an area subject to intense agriculture. Relatively high levels of herbicide residues, notably phenoxy acids (2,4-D and 2,4,5-T), were found in the tissues of stressed corals that had lost symbiotic zooxanthellae and were often in a morbid state. Highest phenoxy acid concentrations reached 8-20 ppb (2,4-D) and 8-19 ppb (2,4,5-T); a combined concentration of 39 ppb phenoxy acids was found in one coral colony. Controlled experiments performed on Pocillopora damicornis in Hawaii, the same species affected in Panama, demonstrated that short duration (24 h) laboratory tests demonstrated drastic effects (tissue death) on corals at 2,4-D concentrations near those found in affected field specimens (0.02 ppm). Although present results demonstrated that herbicides can kill corals at low concentrations and for brief exposures, the herbicides were also found in seawater sampling (El Nino Southern Oscillation) that probably also affected corals adversely.

Introduction

This study on the effects of herbicides on reef-building corals was motivated by the detection of herbicide residues, most notably phenoxy acids (2,4-D and 2,4,5-T), in the tissues of stressed and dying corals on the Pacific coast of Panama. The reef areas were also subject to intense agricultural activities (crops and cattle) and high herbicide inputs. Chiriqui Province has a dominantly humid tropical climate with an annual precipitation slightly in excess of 2,500 mm (Anon., 1975). Coral mortality in the Gulf of Chiriqui, Panama, which encompasses a sector of the Panamanian Pacific shelf, close to the continent, was observed. Temporal and spatial overlap of 1,000 km (Fig. 1), typically occurred 5-6 weeks after affected corals lost their zooxanthellae (Clyne, 1983). From 50% to 80% of the total live coral cover died on many of the Chiriqui reefs during January-April 1983.

We report here 1) the concentrations of herbicides and the presence of other pollutants in the tissues of affected Chiriqui corals, and 2) controlled tolerance experiments testing 2,4-D, MCP and Tergitol (a dispersant) on corals in Hawaii. The test species, Pocillopora damicornis (Linnaeus), is present in similar habitats in Hawaii and Panama, and is the most abundant coral that suffered the most severe mortality in Panama. While these observations and experiments are limited in scope, their publication seems justified in view of the scant information available on pesticide effects on coral reefs.
Fig. 1. Gulf of Chiriquí and Chiriquí Province, Panama.
Materials and Methods

Coral tissue extraction

Pieces of coral, 12-25 g wet wt., were broken from eight species (Table 1) of
field specimens in Panama (Isla del Rey Island, Gulf of Chirigui), wrapped immedia-
tely in aluminum foil and stored in preparation for herbicide analysis. Corals wrapped
in aluminum foil were broken into small pieces and transferred to weighed-
250 ml round-bottom flasks. Coral and flask were weighed and 150 ml 1:1 ace-
tone-ether was added. Each sample was spiked with 20 ml of silver in acetone.
The aluminum foil was rinsed with an additional 10 ml of ethyl-ether, added to
the round-bottom flask. The tissue was digested by refluxing gently in a sand-
bath flask for 12 h. The digest was added to a 500 ml separatory funnel. The di-
genesis flask was rinsed with an aliquot of ethyl-ether which was added to the
funnel. The ethyl-ether digest was washed with acid water to remove the acetone
and decanted. The ether extract was poured into a 300 ml round-bottom flask
containing 15 ml distilled water, 2 ml of 37% KOH solution and two to three te-
flon boiling chips. A three ball Snyder column was fitted. The ether was evap-
oparated in a steam bath for 30 minutes. The basic aqueous solution was tran-
sferred to a 125 ml separatory funnel, washed twice with 20 ml portions of
ethyl-ether and the ether was discarded. The aqueous layer was acidified with 2
ml of 25% sulfuric acid and extracted with 20 ml, and then twice with 10 ml of
ethyl-ether. The ether extracts were combined and dried over 1 g of acidified
anhydrous sodium sulfate. Then the extracts were transferred to a 25 ml evapo-
ration tube and 0.5 ml of benzene was added. A Snyder column was fitted and the
contents were concentrated in a water bath. The concentrated extracts were re-
 moved from the water bath and allowed to cool before adding 0.5 ml of 14% boric
trifluoride-methanol esterification reagent. They were again placed in the wa-
ter bath for 60 minutes at 50°C. After removing them from the water bath, 4.5
ml of 5% sodium sulfate solution were added and they were mixed on a vortex mix-
er.

 Florisil chromatography separation

A glass Pasteur disposable pipet was packed with a small wash of pre-extracted
glasswool, one inch of Florisil, one-half inch of anhydrous sodium sulfate and a
thin layer of Fischer-attapulgite by tapping the pipet to ensure uniform packing.
The contents in the evaporator tube were added to a 5 ml volumetric flask and
then the benzene layer was pipetted from the flask and placed on the Florisil pipet
column until all the benzene in the volumetric flask was removed. More benzene
was added to the volumetric flask and the operation was repeated until 5 ml of
sample was collected in an 8 ml vial and covered with aluminum foil and a screw
top. The samples were then analyzed by gas chromatography.

Water extraction

The method used for the extraction of water samples is similar to the coral
tissue extraction except that one 1 of water sample, acidified with sulfuric
acid, was transferred to a two-liter separatory funnel and 100 ml of diethyl
ether was added. The sample was shaken vigorously for two minutes and sufi-
cient time was allowed for complete phase separation. The lower aqueous layer
was drained and the upper ether layer was poured into a 500 ml Erlenmeyer flask
containing two grams of acidified anhydrous sodium sulfate. The above procedure
was repeated with another 100 ml of diethyl ether. The ether extracts were com-
bined and dried over one gram of acidified anhydrous sodium sulfate for approxi-
mately two hours. The extract was then transferred to a Kugelihn-Danish assembly
consisting of a 250 ml X-0 flask attached to an evaporative concentrator tube
containing two teflon boiling chips. The extract was concentrated to 5 ml in a
water bath and the same esterification and chromatographic procedure was carried
out as for the corals.

Gas chromatography

A tracoc gas chromatograph Model 222 equipped with 2 nickel-63 electron cap-
ture detectors was employed and nitrogen was used as the carrier gas. A 6 ft
glass column (2 mm ID) packed with 16% SE30 and 6% OV101 on 100-120 mesh Supelco
was used. Gas flow at 45 ml per minute, column temperature at 180°C, detector tem-
perature at 250°C and the inlet temperature at 210°C were maintained.
Energy dispersive X-ray analysis

Oven-dried (60°C) corals were examined for the presence of heavy metals using energy-dispersive X-ray (EDX) analysis. All analyses were done within one week of coral collection. The oven-dried samples were ground further. Small flat-surface specimens of less than 1 cm² were selected for EDX analysis. Each sample was mounted on a carbon stub with an isopropyl alcohol-based glue. They were sputter-coated first with a gold film of about 500 Å thickness followed by a graphite coating. An empty stub was coated identically to serve as the control sample and its EDX spectrum served as the background spectrum. The sample was placed in a scanning electron microscope and examined with an electron beam of 5 kV. A Gill (drifted) detector was used to collect the emitted X-rays and a Keve 704S EDX System was used for data analysis and element identification. The data analysis included the normalization of the X-ray spectrum of the sample by the background spectrum followed by its subtraction. The gold K-series X-ray peak at 2.14 keV was used as the normalization peak. Scanning time was about 200 seconds.

Few data are available on detection limits for EDX analysis. The reported value for Na is 0.795 wt %, while it is 0.005 wt % for Ca (Goldstein et al., 1981). EDX sensitivity to heavier elements is much higher than that for Na and Ca. The detection limits for these elements using atomic absorption spectrometry are 0.0005 and 0.0005 μg/ml respectively. For an elemental analysis by atomic absorption using a 100 μg sample dissolved in 10 ml of solvent, and assuming that the coral organics make up less than 0.01% of the total weight sample on a dry weight basis, the atomic absorption detection limit would be 0.03 and 0.05 μg/ml for Na and Ca in the coral tissue. The observed differences in detection limit for the two methods only applies if one is interested in elemental composition of the entire sample. In the case of corals our interest was in elemental composition of the coral tissues and the outer layer of coral skeleton. Because the EDX method is able to examine only those specific areas of interest, the effective sensitivity of the method is much higher than it appears. Two additional advantages of using the EDX technique are: 1) it can determine if an element is localized in a specific spot (as a contaminant) or if it is evenly distributed in the sample. This separation is not possible with techniques that require sample dissolution; 2) the EDX results are affected by whether an element is organic or inorganic bound. Frequently the solubility behavior of an element organic bound is different from that observed when it is inorganic bound. EDX is independent of solvent extraction efficiency or limits of compound solubility.

Tolerance tests

A commercial herbicide sold under the trade name of "Weed-B-Off" (marketed by Chevron, EPA Registration No. 229-2399-24) was obtained from a local gardening supply center in Kaneoke, Hawaii. The manufacturer's description of ingredients was: "Diethylamino salt of 2,4 dichlorophenoxyacetic acid (2.25%). Diethylamine salt of 2-(2-methyl 4 chlorophenoxy) propionic acid (0.25%). Inert ingredients 99.50%." The latter compound is also a herbicide and in the formulation is known as MCPA. Although not used as commonly as 2,4-D it is similar in structure and has almost the same toxicity as an LD-50 of 650 as compared to 500 for 2,4-D. This herbicide was selected for study because one of its active ingredients, 2,4-D, was found in relatively high concentrations in affected field corals, and because many of the herbicides in use in Panama contain 2,4-D.

The reef-building coral Pocillopora damicornis served as the test species in the tolerance experiments. Specimens were collected from 1-2 m depth on the fringing reef at Coconut Island, Kaneoke Bay. In initial range-finding experiment (A) was set up to assess the possible toxicity of the herbicide to Pocillopora. Solutions of the herbicide were prepared with filtered sea water to give final concentrations of the 2,4-D amine salt of 100.00, 10.00, 1.00, and 0.1 ppm. MCPA was also present in equal concentrations. Three small colonies of Pocillopora, 4-6 cm in diameter, were placed in 600 ml of each of the solutions for 24 h. The solutions were removed after 12 h. All test solutions and controls were aerated throughout the experimental period.

Based on the results obtained in A above, a second series of exposures at identical concentrations (B) were conducted. For this experiment, pure 2,4 dichlorophenoxyacetic acid was used. Since the solubility of the acid in sea wa-
ter was very low it was necessary to convert the acid to the sodium salt. This was accomplished using a minimum volume of 1 N NaOH to dissolve the acid followed by dilution with sea water. No precipitation of the sodium salt was observed.

Five liters of each of a series of solutions were prepared equivalent to 2,4-D concentrations of 1.00, 0.10 and 0.05 ppm. In order to determine the 'start' and 'finish' concentrations of 2,4-D in the test solutions, one liter samples of each solution, plus controls, were preserved in cleaned 'Falcone' plastic bottles to which 5 ml of concentrated H SO₄ had been added. Three small colonies of Pocillopora (6-10) were placed in each of the test solutions for 12 h and provided with aeration.

At the end of 12 h one coral colony from each test solution, plus control, was wrapped in aluminum foil and frozen for later analysis of tissue concentrations of 2,4-D. The remaining two colonies from each solution were placed in clean running sea water for observation over 7 d.

Based on the results obtained in B, a re-run of 4 was conducted, but using "Weed-Be-Gone" 2,4-D amino salt concentrations of 10.0, 1.0, 0.5 and 0.1 ppm (C). Corals were exposed to herbicide for 24 h then placed in clean running sea water for observation.

As for C above, corals were exposed to "Weed-Be-Gone" 2,4-D amino salt concentrations of 10.0, 1.0 and 0.1 ppm plus controls (D). Water samples were taken for before and after exposure. One coral colony per treatment was wrapped in aluminum foil and frozen for later analysis.

Many herbicides contain wetting agents. It is not known whether "Weed-Be-Gone" contains a wetting agent but it certainly possesses detergent properties. One chemical often used as a wetting agent is "Tergitol NPF" marketed by Union Carbide. This agent is normally used at a concentration of 0.25 to 0.50% by volume in the herbicide. A 0.50% solution was made up in sea water and a series of dilutions made with sea water to give concentrations of 25.0, 2.50 and 0.25 ppm Tergitol. These dilutions were the same as those used for "Weed-Be-Gone" to give concentrations of 10.0, 1.0 and 0.1 ppm of the 2,4-D amino salt. Three colonies of Pocillopora were exposed to each solution, plus control, for 24 h, then transferred to clean sea water. All samples received aeration during exposure to the detergent.

Two tests were performed with "Tergitol NPF" at low concentration (0.025 ppm) and elevated sea water temperatures. Each group of coral (3 colonies) was held in 1 l of aerated sea water. The first test lasted 24 h and comprised the following treatments: 1) Tergitol and normal sea water temperature (25 - 27 °C), 2) high variable sea water temperature, elevated from 25.7 °C to 34.5 °C over a 4 h period, and then allowed to cool gradually to ambient temperature, 3) Tergitol and high variable sea water temperature (as in 2 above). The second test lasted 66 h and comprised the following treatments: 1) Tergitol and normal sea water temperature (25 - 27 °C), 2) high, relatively constant sea water temperature (23.2 - 31.9 °C), 3) Tergitol and high sea water temperature (as in 2 above). The corals were transferred to tanks with running sea water after each test and their condition observed for 1 week.

Results

Coral tissue analyses

Phenol acid concentrations of 40 ppm were detected in four of the eight coral species analyzed (Table 1). All bleached corals contained relatively high concentrations of herbicide and 2,4-D was present generally in higher amounts than 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) in 9 of 12 specimens. However, some species that appeared in normal condition, e.g., Pocillopora damicornis and Pocillopora pulex (Werrill), had relatively high concentrations of phenol acids, whereas partially bleached or bleached corals had lower amounts. No herbicides were found in an apparently normal coral (Pocillopora damicornis) sampled in the Gulf of Panama (Fig. 1), but a partially bleached coral, (Pocillopora verrill) sampled in Chiriqui was also herbicide-free.

Insecticides found in Panamanian (Pacific) coral tissues included P, P' DDT, O,P' DDT, P,P' DDE; Lindane, Endrin ketone 153, Endrin, Dieldrin, Ethion, Endosulfan I, Chlordecone and Dimethoate. Two plasticizers were also identified, DBP (butyl benzyl phthalate) and DEEP <di-(2-ethylhexyl) phthalate>.
<table>
<thead>
<tr>
<th>Coral species</th>
<th>Condition</th>
<th>ppm concentrations (ng/g dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Millepora intricata</em></td>
<td>bleached</td>
<td>15.850</td>
</tr>
<tr>
<td><em>Milne-Edwards</em></td>
<td></td>
<td>2.050</td>
</tr>
<tr>
<td><em>Pocillopora damicornis</em></td>
<td>normal</td>
<td>20.050</td>
</tr>
<tr>
<td></td>
<td>partially bleached</td>
<td>1.330</td>
</tr>
<tr>
<td></td>
<td>bleached</td>
<td>7.729</td>
</tr>
<tr>
<td></td>
<td>normal⁴</td>
<td>ND²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td><em>Pocillopora elegans</em></td>
<td>partially bleached</td>
<td>1.290</td>
</tr>
<tr>
<td>Dana</td>
<td></td>
<td>0.376</td>
</tr>
<tr>
<td><em>Favina clavus</em></td>
<td>partially bleached</td>
<td>2.920</td>
</tr>
<tr>
<td>Dana</td>
<td></td>
<td>0.640</td>
</tr>
<tr>
<td><em>Psammechinus varians</em></td>
<td>normal</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td>partially bleached</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>bleached</td>
<td>16.710</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.870</td>
</tr>
<tr>
<td><em>Gardineroceros planulata</em></td>
<td>partially bleached</td>
<td>4.326</td>
</tr>
<tr>
<td>Dana</td>
<td></td>
<td>2.840</td>
</tr>
<tr>
<td><em>Porites panamensis</em></td>
<td>bleached</td>
<td>8.630</td>
</tr>
<tr>
<td>Terrill</td>
<td></td>
<td>1.160</td>
</tr>
<tr>
<td><em>Pachyseris stellaris</em></td>
<td>normal</td>
<td>12.080</td>
</tr>
<tr>
<td></td>
<td>partially bleached</td>
<td>2.690</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.973</td>
</tr>
</tbody>
</table>

⁴Collected at Melones Island, Gulf of Panama, 5 May 1983
⁵None detected.
In the EDX analysis, Ca and Cl were the two most widely found elements in both skeletons and tissues of eight coral species examined (Table 2).

Herbicide tolerance tests

In the range-finding experiment with "Weed-B-Gon" (A), considerable mucus production by the corals was observed within three hours of exposure to herbicide concentrations of 1.0 ppm and greater. Corals in 0.10 ppm 2,4-D were producing mucus after 12 to 18 h of exposure. The process of tissue loss appeared to result in the release of polysaccharides from the calices. The condition of the test corals after 24 h exposure is given in Table 3.

Exposure of Porcellanops to 'pure' 2,4-D (sodium salt) in concentrations ranging from 0.1 to 1000 ppm (B), showed no apparent effects. After seven days all coral colonies were of normal appearance, were very mobile and possessed zooxanthellae. Settlement of the planulae was not observed.

In light of the results from (B), a repeat of experiment (A) was conducted using concentrations of the "Weed-B-Gon" 2,4-D amine salt ranging from 0.1 to 100 ppm (C). All corals suffered complete tissue loss within 24 h (Table 4). The mechanism of tissue loss was similar to that observed in (A) with apparent polyp bail-out (Samarco, 1982) although 48 h elapsed before coral death occurred compared with 24 h in (A). Considerable amounts of mucus were found in the water containing dead corals. Planulae released from corals were not normal and possessed zooxanthellae. Settlement of the planulae was not observed.

Results from experiment (B) were similar to (C) in so far as planulae were released by corals in all test solutions but not by controls. Coral death, however, was recorded only at the highest concentration of "Weed-B-Gon" (1000 ppm 2,4-D amine salt) although corals in 1.0 ppm 2,4-D amine salt appeared to be stressed and the 0.1 ppm 2,4-D amine salt solution was turbid (Table 5). The initial, before and after concentrations of 2,4-D and MCPA in the test solutions containing corals are given in Table 5. Initial concentrations of herbicide solutions and the actual concentrations found in the water at the beginning and termination of the experiment are shown. The differences in concentration between initial and before represent the loss to the glassware, and the differences in concentration before and after represent the loss primarily due to uptake by the coral. Although the MCPA also had initial concentrations from 0.1 to 10.0 ppm, the actual concentrations indicate that most of this herbicide was probably lost to the glassware.

The corals were exposed to different concentrations of 2,4-D (10 2,6,4 and 2,4-D (CMF2), and the extent of accumulation of these herbicides is given in Table 7. The various concentration found in coral tissues was 0.177 ppm 2,4-D (CMF2) at an initial concentration of 10.0 ppm. An increase in the initial concentration of herbicide, from 0.1 to 10.0 ppm, did not necessarily increase the uptake by the coral. MCPA was not detected in the corals but was detected in the test solutions. Since none was detected in the corals, this also indicates that it was absorbed to the glassware.

The Tergitol NPX dispersant showed an adverse effect on corals at a concentration of 0.25 ppm. All corals were dead after 2 d when exposed to this dispersant for 24 h (Table 8). Corals exposed to a low concentration of Tergitol (0.025 ppm) and high temperatures were still alive and appeared normal after 1 week. In the experiment with a high variable temperature, all corals planulated and the polyps remained expanded during the 24 h test period. In the 66 h experiment, a combined treatment with Tergitol and elevated temperature, one coral whitened slightly and retracted its polyps.

Discussion

It is significant that relatively high phenoxy acid concentrations, in the range of 10-20 ppm, were found in coral tissues in Panama, especially in an area of high insolation, strong tidal flux and warm sea temperatures. Such environmental conditions tend to hasten the breakdown of phenoxy acid herbicides. The presence of these stable chemicals, as well as thiourea and Dithene, suggests a large and/or constant source of the material entering the reef areas.

In conclusion, seven approved herbicides whose main active ingredients are pheno-
Table 2. Elemental composition of corals collected at Gwa Island, Gulf of Chiriqui, 28 April 1983, based on EDX analysis.

<table>
<thead>
<tr>
<th>Coral species</th>
<th>Condition</th>
<th>Sample</th>
<th>Element</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pocillopora intricata</em></td>
<td>bleached</td>
<td>bulk</td>
<td>Ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca</td>
</tr>
<tr>
<td></td>
<td>dead</td>
<td>bulk</td>
<td>Ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca</td>
</tr>
<tr>
<td><em>Pocillopora pistillata</em></td>
<td>normal</td>
<td>bulk</td>
<td>Ca, Sr (7) or Si</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca, Cl, S, P, K, Mg</td>
</tr>
<tr>
<td></td>
<td>dead</td>
<td>bulk</td>
<td>Ca, Sr (7) or Si</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca, Cl, S, P, K, Mg, Na, Zn (7)</td>
</tr>
<tr>
<td><em>Pocillopora elegans</em></td>
<td>bleached</td>
<td>bulk</td>
<td>Ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca, Cl, Mg, Na, Si, K</td>
</tr>
<tr>
<td><em>Porites gigantea</em></td>
<td>normal</td>
<td>bulk</td>
<td>Ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca, Cl, Mg, Fe, Si, Na, K</td>
</tr>
<tr>
<td><em>Porites clavis</em></td>
<td>bleached</td>
<td>bulk</td>
<td>Ca, Cl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca, Mg</td>
</tr>
<tr>
<td><em>Parazoanthus versicolor</em></td>
<td>partially bleached</td>
<td>bulk</td>
<td>Ca, Mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca, Cl, Mg, Fe</td>
</tr>
<tr>
<td><em>Gardineromorpha planulata</em></td>
<td>partially bleached</td>
<td>bulk</td>
<td>Ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td>Ca, Cl, Si, Mg</td>
</tr>
</tbody>
</table>

1 Broken skeletal face, 2-3 mm below colony surface.
2 Colony surface, with adhering tissues except in two dead specimens.

Table 3. Results of range-finding tolerance of *Pocillopora damicornis* to "Reed-B-Con" (A).

<table>
<thead>
<tr>
<th>2,4-D amine salt concentration (ppm)</th>
<th>Condition of corals after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0</td>
<td>Dead; complete loss of tissues from skeleton.</td>
</tr>
<tr>
<td>10.0</td>
<td>Dead; tissues sloughing off; large areas of bare skeleton.</td>
</tr>
<tr>
<td>1.0</td>
<td>Dead; complete loss of tissues from skeleton.</td>
</tr>
<tr>
<td>0.1</td>
<td>Morbid; tissues sloughing off; small areas of bare skeleton.</td>
</tr>
<tr>
<td>control</td>
<td>Appeared normal; polyps open.</td>
</tr>
<tr>
<td>2,4-D salt concentration (ppm)</td>
<td>Condition of corals after 24 h</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>10.0</td>
<td>Appeared normal; many planulae released.</td>
</tr>
<tr>
<td>1.0</td>
<td>Appeared normal; 10-20 planulae released.</td>
</tr>
<tr>
<td>0.5</td>
<td>Appeared normal; 1-2 planulae released.</td>
</tr>
<tr>
<td>0.1</td>
<td>Appeared normal; no planulae released.</td>
</tr>
<tr>
<td>Control</td>
<td>Appeared normal; no planulae released.</td>
</tr>
</tbody>
</table>

Table 5. Condition of *Pocillopora damicornis* after exposure to "Feed-B-Gon" (D).

<table>
<thead>
<tr>
<th>2,4-D salt concentration (ppm)</th>
<th>Condition of corals after 24 h</th>
<th>Condition of corals after 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>Appeared normal; many planulae released.</td>
<td>Dead; complete loss of tissues.</td>
</tr>
<tr>
<td>1.0</td>
<td>Appeared normal; many planulae released.</td>
<td>Appeared stressed; water very turbid.</td>
</tr>
<tr>
<td>0.1</td>
<td>Appeared normal; many planulae released.</td>
<td>Appeared normal; water slightly turbid.</td>
</tr>
<tr>
<td>Control</td>
<td>Appeared normal; no planulae released.</td>
<td>Appeared normal; many planulae released.</td>
</tr>
</tbody>
</table>
Table 6. Herbicide concentrations in laboratory test solutions.

<table>
<thead>
<tr>
<th>Initial concentration (ppm)</th>
<th>Compound</th>
<th>Concentrations (ppb) before</th>
<th>after</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.1 2,4-D</td>
<td>3.29</td>
<td>2.52</td>
<td>-</td>
</tr>
<tr>
<td>MCPP</td>
<td></td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>1.0 2,4-D</td>
<td>60.78</td>
<td>4.97</td>
<td>-</td>
</tr>
<tr>
<td>MCPP</td>
<td>1.37</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10.0 2,4-D</td>
<td>309.53</td>
<td>95.67</td>
<td>-</td>
</tr>
<tr>
<td>MCPP</td>
<td>13.61</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Not detected.

Table 7. Herbicide concentrations detected in laboratory test coral tissues.

<table>
<thead>
<tr>
<th>Initial Concentration (ppm)</th>
<th>2,4-D MCPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>2,4-D Na+</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>2,4-D (CONH2)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
</tr>
</tbody>
</table>

*Not detected.

Table 8. Condition of Pocillopora damicornis after exposure to the wetting agent Tergitol NPX.

<table>
<thead>
<tr>
<th>Tergitol NPX concentration (ppm)</th>
<th>Condition of coral after 24 h</th>
<th>Condition of coral after 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>75.0</td>
<td>Dead; extensive loss of tissue; water green</td>
<td>Discontinued</td>
</tr>
<tr>
<td>2.50</td>
<td>Polyps withdrawn; water turbid</td>
<td>Dead; extensive loss of tissue</td>
</tr>
<tr>
<td>0.25</td>
<td>Appeared normal</td>
<td>Dead; extensive loss of tissue</td>
</tr>
<tr>
<td>control</td>
<td>Appeared normal</td>
<td>Appeared normal</td>
</tr>
</tbody>
</table>

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noxy acids. The amino salt of 2,4-D is the chief component in mix of the herbicides, and the seaweed (DEF) contains both 2,4-D and 2,4,5-T.

Chiriqui Province, which borders the Gulf of Chiriqui to the north and west, is a highly productive farming region in Panama. In absolute terms, Chiriqui and Veraguas (immediately east of Chiriqui) Provinces have the largest land areas under exploitation (Anonymous, 1975.) Five of the principal crops in Chiriqui in 1963, and the respective areas under cultivation, were corn, 2,000 ha.; sugar cane, 5,000 ha.; sorghum, 5,000 ha.; bananas, 7,000 ha.; and rice, 22,000 ha. (Gobierno de Panama, Departamento de Agricultura, personal communication). Herbicides are applied regularly to these crops by aerial spraying. The recommended doses are slightly below 11 ha−1. Moreover, it is highly probable that the recommended doses have been exceeded in some areas. Claims have been made by local farmers that crop dusters dump excess herbicides directly into the Gulf of Chiriqui before landing.

The universal presence of Ca in the elemental analysis of surface and bulk samples is probably due to the high calcium carbonate content of the coral skeleton. Chlorine was also commonly present, mainly in surface tissue, and could be due to natural salts and chlorinated pesticides in coral tissue. There was no evidence of heavy metal contamination based on EDX examination.

Some of the laboratory tolerance tests demonstrated dramatic effects on corals at 2,4-D concentrations near those found in the tissues of affected field specimens (0.02 ppm). Coral tissue sloughing and death occurred after exposure to the herbicide "Weed-B-Gone" (in 2 of 3 experiments) at a concentration of 0.1 ppm 2,4-D amino salt. Since these laboratory exposures were of short duration, not exceeding 24 h, it is possible that longer exposure at lower concentrations would also cause morbidity and death in corals.

The variable response of Pocillopora to similar concentrations of "Weed-B-Gone" in different experiments may be attributable to the changing physiological state of the corals. Pocillopora has a monthly planulation cycle and an experimental period of two weeks. Release of planulae but not controls in the latter two experiments, and the progressively longer time required to result in coral death at 0.1 ppm 2,4-D amino salt, suggest that changes associated with reproductive cycle may have influenced coral response to the herbicide. This may prove to be an important consideration in future toxicological studies on corals.

Two herbicides tested on Hawaiian Pocillopora damicornis by Lambert (1983), at low concentrations (0.01-2.0 ppm) for 24 h, produced no evidence of injury. One of the herbicides tested was 2,4-D. Since concentrations were not reported in affected field corals, from an extensive kill of unknown cause in American Samoa, or in the test water and tissue in the laboratory studies, it is difficult to compare Lambert's results with those reported here.

It should be noted that the toxic herbicide concentrations detected in the laboratory test coral tissues were notably lower than the initial concentrations. Also, since the SRP herbicide tended to absorb to the glassware, it can be concluded that the killing effect was due largely to 2,4-D.

Because pure 2,4-D produced no apparent effect over a range of concentrations (0.1 to 10.0 ppm), it was reasoned that a wetting agent may also adversely affect corals. Tergitol NP-10, a commonly used dispersant, did cause coral death at 0.25 ppm. Tergitol at a lower concentration (0.025 ppm), and in combination with high temperatures (32-34.5 °C), produced no apparent damage to corals. This result was unexpected because previous studies in Hawaii have shown that elevated temperatures alone, from 4 °C to 5 °C above ambient to 12°C, causes death in Pocillopora damicornis in 3 to 5 days (Wool and Coles, 1974; Coles et al., 1976; Wijek and Coles, 1977).

While present experimental results demonstrate that herbicides can have deleterious effects on corals, at relatively low concentrations and for brief exposures, the coral mortality that occurred recently in Panama and elsewhere in the eastern Pacific (Glyn, 1984; in preparation) cannot be attributed to herbicide effects alone because of other confounding factors. The tropical eastern Pacific region was subjected to a very strong El Niño event during 1982-83, probably the strongest this century in terms of equatorial sea surface warming (Kerr, 1983). The warm water anomaly exceeded 2 °C over large areas of the eastern equatorial Pacific and even reached 6 °C in places (Rasmussen and Ball, 1983).

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It can be reasoned that the high levels of herbicides in Chiriquí corals, in
combination with an unusually prolonged warming spell, could act additively and
thus lead to coral mortality. However, massive coral death also occurred in the
Galápagos Islands, in coincidence with the 1982-83 El Niño, but in this area
pesticide use is nil. In the light of these results, coral death and El Niño
severity are not all. In the light of these results, coral death and El Niño
weakening show the strongest correlation. Still, the high levels of phenolic acids
present in affected field corals, and the demonstrated laboratory effects, make
it difficult to dismiss the possibility that herbicides also played a role in
the Chiriquí coral death.

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Isolation and culture of symbiotic algae

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Abstract

Techniques for the isolation and culture of symbiotic algae are described. Freshly collected invertebrates are cleaned with sodium hypochlorite before removal of symbionts. The symbionts are suspended in clean seawater, passed through filters, and isolated with modified Pasteur-type pipets under stereo microscopes. Clone cultures have been the most successful and have been maintained for years.

Introduction

Several methods for isolating microscopic algae are described in chapters in The Handbook of Physiological Methods (Stein 1973). Other chapters in the same text discuss media, sterilization and culture. Using a modification of the Pasteur-type pipet technique, clones of symbiotic algae have been isolated from various marine invertebrates and added to the Algae Culture Collection of the University of Hawaii. After learning this technique during the 1983 Summer Program in Marine Science at the Hawaii Institute of Marine Biology, Larrie Read successfully isolated and cultured clones of 

Pocillopora damicornis.

Preparation

Pasteur pipets (23 cm long) are stretched thin by melting the narrow section over an alcohol burner flame and pulling on the ends as they are removed from the flame. After cooling, they are bent until they break at the stretched part. The inside diameter should be about 100 microns at this point. The other end is plugged with cotton. The pipets are autoclaved in a cylinder that is also cotton plugged. All flasks, pipets, and test tubes are cleaned with laterator detergents, thoroughly rinsed with distilled water, rinsed with 10% hydrochloric acid, rinsed again with distilled water, cotton plugged and autoclaved. The filtration apparatus and dissecting instruments are autoclaved. Seawater and nutrient stock solutions are filter-sterilized through 0.22-micron pore size polycarbonate or membrane filters and stored in fluorocarbon (e.g. Teflon), polycarbonate, boro-silicate glass flasks. These can be stored at room temperature or refrigerated, except for the vitamin stock solutions which should be frozen. Sterilization by autoclaving may be more reliable but will alter the water chemistry, so in this case fluorocarbon flasks are preferred. The nutrients are added to a seawater flask to make 1× medium (Guillard and Ryther 1962). Ten milliliters of medium is dispensed to each 15-ml clear polycarbonate or boro-silicate glass test tube.

Isolation

Sodium hypochlorite is added to one of the sterile seawater flasks to make a 30 micromolar solution (e.g. approximately 1 drop Chlorox/250 ml). A few grams of animal tissue containing symbiotic algae are placed in the hypochlorite solution. After soaking for a few minutes, a small piece of tissue (e.g. branch tip, tentacle, etc.), is cut with a scalpel, and transferred by forceps to new hypochlorite solution. Small invertebrates, or reproductive products (e.g. planulae) are not cut. After two hours, the tissue is transferred by forceps to sterile seawater, agitated and transferred to a sterile petri dish (Falcon 1005) that is half filled with sterile seawater. The tissue is held with the forceps, while it is sliced and washed with the scalpel. The cover is tightly placed on the petri dish which is then agitated. The suspension is poured into a test tube until it is about one-half full, sealed with a sterile silicon stopper, and shaken vigorously as possible. This is poured through a sterile 20 micron mesh screen (e.g. Nitex) into a sterile petri dish and examined microscopically for the presence of algal cells. A stereo microscope is used for isolation. At 100× magnification 10 micron size cells such as 

Porites can be discerned.
If the algae are more concentrated than one to a few cells per field of view at low magnification (20-40x), the suspension is diluted with sterile seawater cells in the field of view at 100x magnification. After, a single cell is located with no other magnification. While holding one finger against the large end of the Pasteur pipet, the small end of the pipet is placed next to the algae cell using one or both hands to hold the pipet. After the finger is removed from the end of the pipet, the flow of water should draw the cell into the pipet.

The tip of the pipet is then placed against the inside wall of one of the test tubes containing medium, and the cell is transferred by blowing into the other end of the pipet. The cotton plug is replaced, and the tube is swirled to wash the cell into the medium. A new pipet and test tube are used each time the procedure is repeated. In some cases, as many as 100 tubes may be necessary for a few successful colonies.

Culture

The tubes are placed 20 cm from fluorescent light bulbs (e.g. Vita-lite, or Cool White). After blooming, the cultures can be maintained by transferring to new medium in test tubes, Erlenmeyer flasks, or other vessels monthly. Cultures of *G. bacilliforme* have survived years in the same flask when tightly capped to prevent evaporation.

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