Helgoländer wiss. Meeresunters. 17, 188–199 (1968)

Toxicological investigations in an artificial ecosystem. A progress report on copper toxicity towards algae and daphniae

HENDRIK J. HUECK and DOROTHEA M. M. ADEMA

Central Laboratory TNO, Delft, Netherlands

KURZFASSUNG: **Toxikologische Untersuchungen in einem künstlichen Ökosystem. Ein Fortschrittsbericht über die Giftigkeit von Kupfer gegenüber Algen und Daphnien.** Eine toxikologische Forschungsarbeit über Probleme der Meerwasserverschmutzung sollte langfristige Experimente anstellen, die sich mindestens über eine Generation der Testorganismen erstrecken, sowie Transport und Anhäufung von toxischen Stoffen möglichst bei den Species untersuchen, die zusammen eine Nahrungskette bilden. Erwünscht wäre auch die Feststellung der Auswirkung zusätzlicher Belastungen (zum Beispiel Hunger, Hitze, Radioaktivität, Säure oder Basen, Mangel an O_2). Dieser Versuchsbericht beschreibt die Einrichtung eines Labortestsystems, mit dem die beiden erstgenannten Ziele angestrebt bzw. verwirklicht werden. Für die Dauerflußexperimente wurde eine künstliche Nahrungskette geschaffen, die aus einzelligen Algen, kleinsten Schalentieren und Fischen bestand, so daß jede Gruppe in das Experiment eingeschaltet wurde. Einige Ergebnisse werden berichtet. In einem Vergleich von Experimenten mit stehendem Wasser, zwischendurch erneuertem Wasser und von Kulturen im Durchfluß mit *Daphnia magna* wird gezeigt, daß Versuche unter Dauerfluß ein besseres und empfindlicheres Verfahren darstellen im Vergleich zu den beiden anderen Verfahren. Als Anhaltspunkte für die Beurteilung der Ergebnisse werden Sterblichkeit und Vermehrungskapazität zugrunde gelegt. Gerade letztere unterliegt am stärksten der Giftwirkung. Die verwendete Alge *Chlorella pyrenoidosa* speicherte in ihren Zellen erhebliche Kupfermengen. Bei *Daphnia magna* war die Konzentration, welche Wachstum verhinderte, etwa 56 p. p. b. Dagegen vertrug *Chlorella pyrenoidosa* Konzentrationen bis 1000 p. p. b. Wenn *Daphnia magna* mit Algen gefüttert wurden, die in kupferhaltigen Medien gewesen waren, so starben sie nur, wenn die Medien 560 p. p. b. enthielten. Es scheint also, daß – trotz der Akkumulation von Kupfer in diesen Algen – eine Vergiftung durch Nahrung weniger wirksam ist als die direkte Vergiftung durch Kupfer im Medium. Die Futtermenge muß gleichfalls berücksichtigt werden. Provisorische Kalkulationen zeigen, daß die in der einzelnen *Daphnia* nachweisbare Kupfermenge beim Futterexperiment etwas höher ist.

INTRODUCTION

Problems connected with the increasing need for disposal of industrial and domestic waste into the North Sea have drawn the attention of scientists in the Netherlands already for some time (DE WOLF 1965). As a result of a tentative distribution of work among several laboratories and institutes concerned, the Central Laboratory TNO began to study the toxicological aspects of marine water pollutants.

For this purpose, we wish to establish a laboratory system in which we may introduce, under more or less defined conditions, the different pollutants coming under investigation. It appears furthermore desirable to check the toxic effects of pollutants, either in field experiments or under semi-natural conditions when field experiments are impractical.

In this contribution to the International Symposium of the Biologische Anstalt Helgoland we shall discuss the guiding principles adopted for the laboratory system, together with some preliminary results which we have obtained with copper as a toxic agent.

AIMS OF THE INVESTIGATION

In toxicology, a sharp distinction should be made between acute toxicity and chronic toxicity. It is inherent to pollution problems that the chronic toxicity due to low concentrations of the pollutant is of great importance. This calls for tests of long duration, preferably covering one or more generations of the test organisms. In the marine environment, as in most other environments, the interrelations between different species, as in food chains, are very important.

A toxicological testing system, therefore, should cover a number of species that are representatives of different large taxonomic groups and, if possible, it should take into account interspecific relations. Studies on the fate of radio-active waste in the marine environment (POLIKARPOV 1966), and recent experiences with non-degradable pesticides and surfactants, have shown the importance of transport and accumulation of toxic substances in and between organisms (ROBINSON et al. 1967). A test system, therefore, should allow one to take these phenomena into account, if possible. Last, but not least, we must mention – as a special feature of the problem we are facing – that pollutants are hardly ever pure substances, readily definable in chemical terms. We generally have to deal with mixtures of toxic substances, which, moreover, may be accompanied by substances or conditions (e.g. organic waste, leading to a lowering of oxygen tension; starvation; heat; radio-activity; acid or basic substances) and which – though not in themselves directly toxic or lethal – may provide a secondary stress to the organisms in question. Taking these considerations into account, we have set ourselves as a goal the development of a test system that has the following features: (1) the system should allow for long-term experiments, involving at least one generation of the test organism; (2) the system should involve several species of test organisms, preferably linked in a food chain; (3) the system should allow for the application of secondary stress. So far our efforts have been mainly concentrated on the first two items, and they will be discussed here further.

Figure 1A shows the simple situation of an aquarium experiment with one organism only. If more organisms are to be investigated, one could think of a simple multiplication of this design as shown in B. In that case, however, the interspecific relations, as e.g. prevalent in a food chain, are not taken into account. A design without this drawback is given in Figure 1C. Such a set-up, (the „microcosm" technique), however is hardly conducive to an accurate analysis of what is happening, both

chemically and biologically, as is necessary in toxicological tests. A way out is shown in design D, where each organism has its own compartment but is connected by overflow, or similar devices, with its predecessors in the food chain. Such a device calls for continuous culture or, at any rate, for continuous-flow experiments.

A set-up like this would also be favourable to the first requirement, namely, the necessity of carrying out long-term experiments. In acute toxicity experiments of short duration, static tests are generally used. Such tests are useful for supplying a

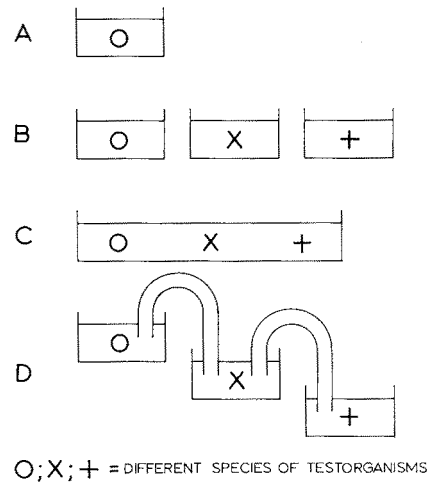

Fig. 1: Possible schemes of toxicity tests with aquatic animals. (Further explanations in text)

specified dose to a test organism. Our problem, however, is to investigate the exposure of test oganisms to a certain environmental concentration of the toxic agent (MOUNT & WARNER 1965). In static tests, the concentration of the toxic agent rapidly decreases through absorption by the test organism and the apparatus, through breakdown, evaporation, precipitation, etc. To maintain controlled conditions, especially the level of the toxic agent, use of continuous-flow tests appears to be highly desirable (HUECK & ADEMA 1967). Accordingly, we have chosen to work with continuous-flow cultures using as test organisms unicellular algae, micro-crustaceans and fishes in the experimental set-up of design D in Figure 1. To establish the feasibility of such a system, we are working for the moment with freshwater species in order to avoid the difficulty of the (continuous) cultivation of marine organisms.

It is our intention to switch to truly marine organisms next year, when better facilities for this work will be available. It will be appreciated that for a comparison of the merits of static and continuous-flow experiments and the importance of interspecific relations, as will be reported in the experimental part of this paper, use of freshwater test organisms is convenient and permissible.

MATERIALS AND METHODS

In the following we will give some information on toxicity tests with an unicellular alga (*Chlorella pyrenoidosa*, CHICK, University of Wisconsin, strain 2005) and a branchiopod (*Daphnia magna*, STRAUS, own isolation): *Chlorella* was cultured in a synthetic medium (HUECK & ADEMA 1967), and *Daphnia magna* in a medium according to FREEMAN & FOWLER (1953).

The main criterion used in our evaluation was the assessment of numbers of these organisms. For algae this was done with microscopical counting chambers and, later, with a Coulter-counter. The daphniae were counted by catching them individually with a pipette.

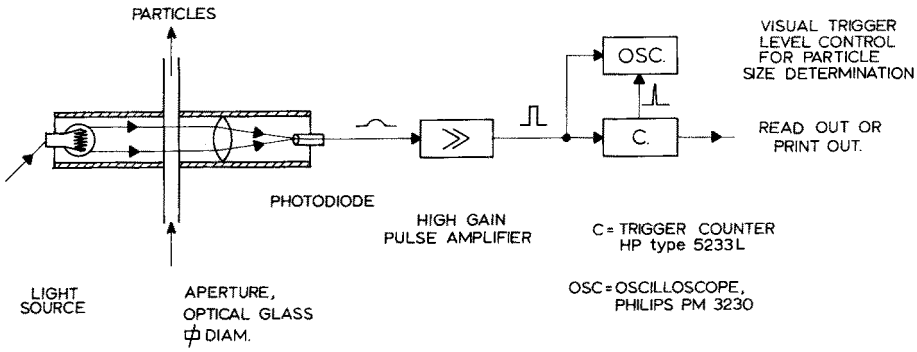

Fig. 2: Photometric counting of particles (daphniae)

Later an apparatus was developed in which daphniae, passing with a flow of water through a counting cell, interrupted a beam of light. The interruptions were counted electronically[1] (Fig. 2). The apparatus is still under development, but has already given some promising results.

As a toxic agent, copper was used as $CuSO_4$. The organisms were maintained in aquaria of different sizes. The flow of water was maintained by peristaltic pumps (LKB type 4912 A).

Most experiments were carried out in a conditioned room at 22° C, in normal daylight. The distilled water used for making up the media was taken from the laboratory main and filtered over carbon (Norit PKX 0.5–1 mm). This filtration step was found to be necessary, as media made up with untreated distilled water proved to be unsatisfactory.

Estimation of copper was carried out colorimetrically with diethyldithiocarbamate (STRICKLAND & PARSONS 1965).

---

[1] The apparatus was developed by Ir. G. A. SCHWIPPERT of our institute. Details will be published elsewhere.

EXPERIMENTAL PART

Introduction

From the many experiments in our investigation, a few are chosen here to illustrate the following points: (a) a comparison of the results of static and continuous-flow toxicity tests with daphniae; (b) food relations between algae and daphniae under influence of copper.

Our investigation as a whole aims at drawing up a budget of copper present in the organisms linked in the artificial food chain under the influence of different levels of this toxic agent in the environment. Apart from the fact that we have not yet completed the picture, the scope of this paper does not allow for an overall discussion of this broad problem.

A comparison of static and continuous-flow toxicity tests with *Daphnia magna*

Toxicity tests with daphniae are generally carried out in two different ways: (1) 30 young daphniae are put in small vessels (300 ml) and kept until they produce a second generation. From the second generation a sample is drawn, with which the experiment is continued.

(2) Five adult females, which are on the verge of spawning, are introduced into a large experimental vessel (10 litres). The offspring produced remains in the experimental

Table 1

Effect of copper in static cultures and intermittently renewed cultures of *Daphnia magna*

Type of culture	Concentration Cu (p.p.b.)	P = parent generation		F_1 = first generation offspring	
		Survival %	Reproduction coefficient	Survival %	Reproduction coefficient
Static culture	0	100	13	89	8
	10	100	4–10	91	7
	18	100	11	86	9
	32	95	9	30	3
	56	27	0	–	–
	100	1	0	–	–
	180	0	–	–	–
	320	0	–	–	–
Culture medium renewed every 3 days	0	99	10	93	8
	10	99	7	46–91	9
	18	100	5	75	5
	32	100	1	63	0
	56	43	0	0	–
	100	0	–	–	–
	180	0	–	–	–
	320	0	–	–	–

vessel, where they produce after some time a second generation. The population is left undisturbed, except for countings at distinct intervals.

As parameters both mortality (complementary to survival) and the production of offspring are used. In general in our blank cultures 5 ripe females produce 3 broods in 9 days, each comprising about 200 young daphniae. The daphniae in the following experiments are fed with algae grown in non-toxic media.

In the experiment summarized in Table 1, the influence of copper in a static test of the design (1) is given: one series is run without any renewal of the test medium while simultaneously in another series the test medium is renewed with intervals of 3 days. The data given are means of duplicate tests. In cases of severe discrepancies between parallels, both data are given. The survival in a certain period was calculated as a „percentage" according to:

$$\text{percentage survival} = \frac{100 \cdot \int_0^{t_f} N_t \cdot dt}{N_0 t_f}$$

where N_t = number of daphniae present at time t

t_f = time of finishing the experiment.

A crude coefficient of reproduction was calculated by dividing the offspring after a certain period by the number of parents present at the date of spawning. The duration of the experiment was about three weeks.

It will be seen from this table that in this experiment the F_1 generation shows a more sensitive reaction towards copper than the P generation. Furthermore, we may

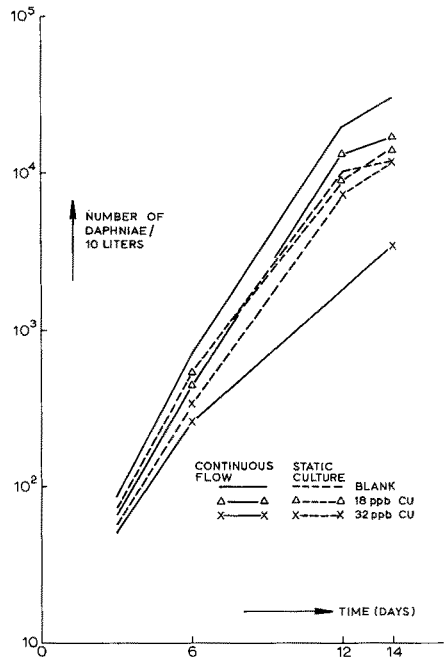

Fig. 3. Toxicity of copper against *Daphnia magna*

note that reproduction shows a tendency towards greater sensitivity to copper. Lastly it can be seen that toxic effects in the renewed media are more pronounced than in truly static media. To investigate this further we carried out an experiment comparing continuous-flow at a rate of 5 l/day in aquaria of 10 l contents, with static cultures (design 2). The result is shown in Figure 3. Though the result is less detailed than that in the previous experiment, it shows clearly that in static culture there is only some initial retardation in the development of the population due to copper but that this levels out in the long run. The final size of the population is apparently determined by the size of our aquaria. This may be explained by exhaustion of the limited amount of copper available through absorption by the organisms. Continuous-flow, on the other hand, allows a denser population to develop in the experimental vessel and gives rise to a clear-cut differentiation of population size under influence of copper. As may be expected from Table 1, no appreciable development of the population occurs on the level of 56 p.p.b. copper; reproduction at that level is fully impaired, though mortality is not quite 100 %.

Even with the flow-rate maintained in this experiment, exhaustion of copper occurs to some extent. Improvement is looked for. At any rate we may conclude that continuous-flow experiments, preferably over two or more generations, provide a more realistic picture of actual toxicity of copper than static experiments.

Food relations between daphniae and algae under influence of copper

Chlorella pyrenoidosa can stand much higher concentrations of copper than daphniae; this can be seen from Figure 4, which shows the result of the development of this alga in static culture under influence of copper. Continuous culture tests are being carried out, but have not yet been completed. We may note again an initial retardation of growth with lower concentrations of copper, but this too levels out in the long run so that the final density reached is the same over the range from 0 to 560 p.p.b. copper. Over the range 560 p.p.b. to 3000 p.p.b. copper (= 3 p.p.m.) production steeply drops to zero. It must be remarked that copper has also some influence on the diameter of the algae, as is shown in Figure 5, which gives means of 50 measurements. The differences are statistically significant, but refer to only one experiment. In these cultures, *C. pyrenoidosa* accumulates considerable quantities of copper.

Provisional estimations carried out by us resulted in a concentration factor of about 4000 to 5000 x. This is somewhat higher than the factors of 100 to 2000 x in data from radio-ecological studies as summarized by POLIKARPOV (1966, p. 78), though they are certainly not unusual in this type of experiment.

We wondered what the effect would be if we fed our sensitive daphniae with algae cultured in media containing different concentrations of copper. We first tried to establish the amount of algae consumed by the daphniae. For the purpose we provided 6 levels of algal food to 10 daphniae in 300 ml beakers. As the daphniae grew during the experiment, and small daphniae cannot be held in too dense algal suspensions, we had to increase the amount of algae fed every two days. Thus we started

with levels of 2.5 × 10^7, 5 × 10^7, etc. algal cells/300 ml on the first day and we ended with levels after 8 days of 40 × 10^7, 80 × 10^7, 160 × 10^7, etc. algal cells/300 ml. Generations were counted separately. The result is shown in Figure 6, in which the median level of feeding (after 6 days) is taken as a parameter representing the level of feeding.

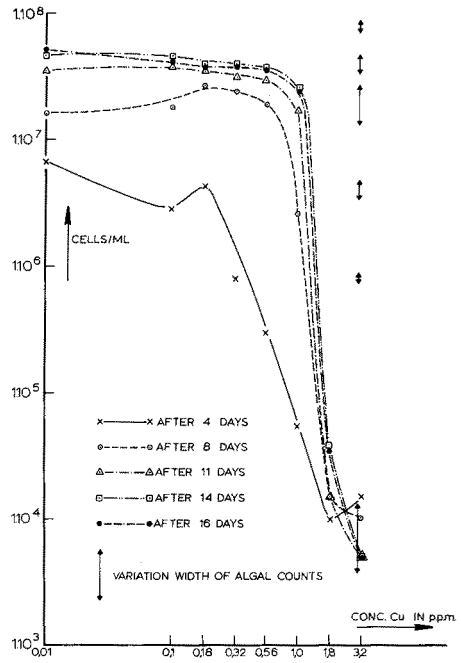

Fig. 4: Development of *Chlorella pyrenoidosa* in static culture in different copper concentrations

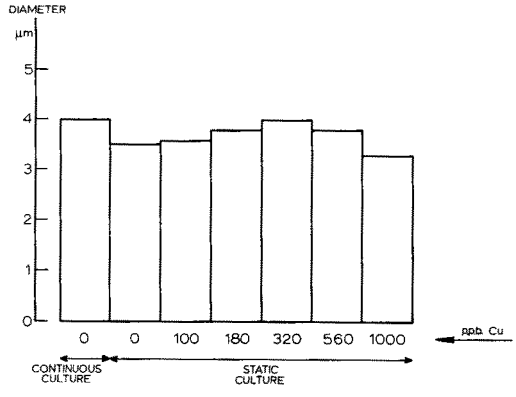

Fig. 5: Diameter of *Chlorella pyrenoidosa* under influence of copper

It will be noted that mortality (survival) is hardly affected. On the other hand, the reproductive capacity shows a clear-cut optimum. There was a tendency for the generation time to be optimum too in the same region. Furthermore, we noted the appearance of white bodies, giving fat reactions, in the daphniae at the three higher levels of feeding. Because of settling of algae in the experimental vessels, we could not

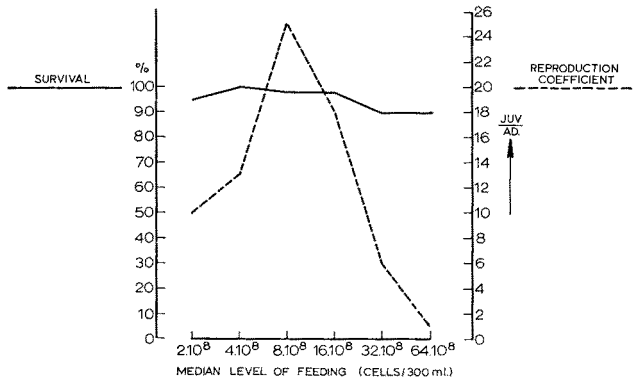

Fig. 6: Influence of different levels of feeding with algae on development of daphniae (10 D/300 ml)

determine exact percentages of algae consumed at the higher levels of feeding. At the lower levels, the algae were consumed practically quantitatively. It could not be decided, in this experiment, whether the unfavourable effect of high levels of feeding was due to overeating or crowding of the experimental vessels with algae. We chose to continue our experiments with feeding the daphniae at level 3.

In the last experiment to be reported here, we cultured algae in media containing different concentrations of copper. The algae thus obtained were fed at level 3 to *D. magna* in a non-toxic medium. The result is shown in Table 2, and compared with the influence of copper dissolved directly in the daphnia medium, and with intermittent renewal of the medium concurrent with the feeding of algae.

In Table 2 the results for survival etc. are not directly comparable with those of Table 1, as we had to finish the experiment at an earlier date. It will be noted, however, that the trend in both experiments is about the same. An inconsistency occurred in the feeding experiment in regard to the parent generation at 180 p.p.b. From earlier experiments, not recorded here, we had noted a consistent drop at 560 p.p.b. so that we suspect an experimental error to have occurred, which does not affect our main conclusion. Though the experiment is not perfect, it shows clearly that poisoning daphniae by way of food is not nearly as efficient as direct poisoning by copper in the medium, even with a suggestive concentration factor of 4000 to 5000 x in *Chlorella* as found by us. We tried to calculate, on the basis of the volume of algal cells consumed and an assumed concentration factor of 4500, how much copper was really available to each daphnia. We next compared this calculated amount with the direct poisoning, assuming in that case a total exhaustion of copper in the medium. The outcome of this calculation is given in column 4 of Table 2. In carrying out this

Table 2

Influence of copper on daphniae via food and via direct effects of copper dissolved in the medium (Juv : juveniles, Ad : adults)

Type of experiment	Initial copper concentration in algal medium in p.p.b.	Initial copper concentration in daphniae medium in p.p.b.	Total amount copper available in 6 days per *Daphnia* in μ gram present P	present F_1	consumed P	consumed F_1	P = parent generation Survival %	P = parent generation Reproduction coefficient $\left(\frac{\text{Juv}}{\text{Ad}}\right)$	F_1 = first generation offspring Survival %	F_1 = first generation offspring Reproduction coefficient $\left(\frac{\text{Juv}}{\text{Ad}}\right)$
Toxic food	0	0	0	0	0	0	90	12	100	14
	100	0	0.45	0.39	0.43	0.39	87	5–10	100	11
	180	0	1.2	1.1	1.1	0.9	75	(4)	97	13
	320	0	2.2	2.2	< 1.6	< 1.7	87	12	90	2–9
	560	0	3.3	3.5	< 1.5	< 3.0	90	2	98	2
	1000	0	7.7	–	< 1.5	–	20	0	–	–
					present					
Toxic medium	0	0			0		100	10	98	6–10
	0	10			0.20		100	5–8	72	9–13
	0	18			0.36		100	3–7	82	2–8
	0	32			0.64		100	1–7	80	0
	0	56			1.12		43	1	3	0
	0	100			2.00		0	–	–	–
	0	180			3.60		0	–	–	–
	0	320			6.40		0	–	–	–

calculation, we found that the interpretation of its results is far more complicated than we expected when first starting the experiment. We wish to reserve further comment for future occasions, and for the present use the data only to show in which way we expect to proceed. In the long run, a complete budget of copper in the organisms and the environment should be attempted.

SUMMARY

1. It is suggested that the toxicological investigation of marine pollution should aim at studying, in long-term experiments, transport and accumulation in food chains and the influence of secondary stress on these phenomena.
2. A laboratory set-up is described with algae, micro-crustaceans and fishes as an artificial food chain kept in continuous-flow systems.
3. Preliminary results with copper as the toxic agent are given with *Chlorella pyrenoidosa* and *Daphnia magna* as the experimental organisms.
4. It is shown that in toxicity experiments assessed by mortality and generation size, continuous-flow tests are more discriminating and sensitive towards *D. magna* than static tests. Reproduction is the more sensitive measure. A concentration of 56 p.p.b. copper is limiting for the development of daphniae.
5. A concentration of about 1000 p.p.b. copper is limiting for the development of *C. pyrenoidosa*. Copper is accumulated in its cells about 4000 to 5000 x.
6. If fed with algae cultured in copper-containing media, it is found that *D. magna* is inhibited at concentrations of 560 p.p.b. copper in the algal medium. It is concluded that poisoning by way of food in this case appears to be less efficient than poisoning directly by way of dissolved copper in the medium.
7. Provisional calculations, taking into account the rate of feeding, suggest that the actual amount of copper becoming available to the daphniae by way of food is slightly higher than that by direct absorption from the medium.

Acknowledgments: The investigations reported above are the result of the work of a research team of the Biological Department of the Central Laboratory TNO. Without the technical assistance of Mrs. D. VAN DRONGELEN, Miss TH. A. M. VAN ZIJL, Miss J. ZUIDWEG, Miss C. W. M. VAN HOLSTEIJN and Miss S. YU the work would have been impossible. Mr. H. COMPAAN provided advice on the chemical analysis of copper, which was carried out by Mr. R. A. F. WINIA of the Analytical Centre of our institute and his staff. Thanks are also due to Drs. P. DE WOLF for his advice on biologial questions and to Mr. G. SCHWIPPERT of the Physics Department of our institute, who, at our request, designed and built an electronic daphnia counter. Last, but not least, we wish to thank our directors for making available the considerable means necessary for this investigation.

LITERATURE CITED

FREEMAN, L. & FOWLER, J., 1953. Toxicity of combinations of certain inorganic compounds to *Daphnia magna* STRAUS. *Sewage ind. Wastes* **25** (10), 1191–1195.

HUECK, H. J. & ADEMA, D. M. M., 1967. Some problems in the testing of materials with algae. *Material Organismen* **2** (2), 141–152.

MOUNT, D. J. & WARNER, R. E., 1965. A serial-dilution apparatus for continuous delivery of various concentrations of materials in water. *Publ. Hlth Serv. Publs, Wash.* No. **999**-WP-23, 16.

POLIKARPOV, G. G., 1966. Radio-ecology of aquatic organisms. North-Holland Publishing Co., Amsterdam, 314 pp.

ROBINSON, J., 1967. Dynamics of organochlorine insecticides in vertebrates and ecosystems. *Nature, Lond.* **215**, 33–35.

— RICHARDSON, A., CRASTRUE, A. N., COULSON, J. C., POTTS, J. R., 1967. Organochlorine residues in marine organisms. *Nature, Lond.* **214**, 1307–1311.

STRICKLAND, J. D. H. & PARSONS, T. R., 1965. A manual of sea water analysis. 2nd ed. Fisheries Research Board of Canada, Ottawa, 203 pp. *(Bull. Fish. Res. Bd Can.* **125**.)

WOLF, P. DE, 1965. Problemen bij de lozing van afval en afvalwater in zee. *TNO-Nieuws* **20**, 924–934.

Discussion following the paper by HUECK & ADEMA

KINNE: I would like to congratulate Drs. HUECK and ADEMA on this very interesting paper. The important biological entities in nature are systems of co-existing forms of life, rather than single species. Hence, a complete assessment of the biological consequences of toxic substances under laboratory conditions must, ultimately, take into account artificial eco-systems consisting of typical and important representatives of the food-energy chain. Another important aspect is that you did not restrict your studies to the ontogeny of individuals but included the second and third generations. In this way it was possible for you to work with fully acclimated organisms, and to check on possible after-effects on subsequent generations. In fact, we have discussed and planned quite similar experiments at the Biologische Anstalt Helgoland. However – as you have already mentioned – this is not easy to do with truly marine organisms. The critical point here is: we must learn to culture marine organisms in such a way that we can be reasonable sure that they are healthy and perform as they would do in the sea. Unfortunately, a lot of work must be done before we are able to handle marine organisms in the same way in which you can handle *Daphnia magna.*

HUECK: Prof. KINNE stressed a very important point: that the culturing of marine organisms is a bottle-neck in our experiments. This calls for cooperation of laboratories which possess the necessary skills in this complicated field of research on marine pollution.