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WAR DEPARTMENT

PHYSICAL SCIENCES DIVISION

CHEMICAL CORPS BIOLOGICAL LABORATORIES

Project No. 4-61-14-001

Report No. 23

1 Apr 1 1952 to 1 November 1952

Project on Marine Biology

Contract No. DA-18-064-CML-471

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3. 6 August - at Rock Slide, 20 buckets yielding approx.
1,650,000 mouse units.

Total mouse units of poison collected during the summer of
1952 - 5,842,250

For the collections on 23 and 26 July, we had the very welcome assistance of Naval personnel obtained through the offices of Capt. Myers and Lt. Comdr. Raison at the Naval Biological Laboratory, Naval Supply Center, Oakland, Calif.

We have found that grinding the mussel livers into acidified ethyl alcohol at the beach as soon as they are removed practically eliminates any loss of poison such as has been observed if the whole livers are allowed to stand for 24 - 48 hours before grinding. Immediate grinding has been made a routine procedure.

Maintaining the pH of the ground livers at pH 3.0 or below also aids in preserving the poison.

No human or animal cases of shellfish poison were reported in California during 1952.

B. Plankton studies

No "Red water" due to *Gonyaulax* has been reported. During the time of highest poison titers, an occasional *Gonyaulax* was seen in water samples taken on Monterey Bay.

II. Laboratory

A. Illumination of the *Gonyaulax* cultures

By the use of light meters, it was found that the foot candles of light reaching the culture vessels was reduced by as much as tenfold when

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bacterial growth, dust, etc. accumulated in the water of the incubation tanks. To control this disturbing factor, aqueous Merthiolate was added to a final concentration of 1:10,000. This antiseptic has kept the water clear for a period of six months with no evidence of a reduction of light transmittance.

From experiments performed, it appears that fluorescent light is as good if not slightly better than incandescent light as measured by growth rates of *G. catanella* cultures. Fluorescent light has the further advantages of more even distribution of light and less heat produced. In view of these findings, fluorescent lights are being mounted on the small (15 gal.) culture tank.

The 20 liter culture bottles are receiving 350-370 foot candles of light from each of the 2 sets of fluorescent lights under the present conditions.

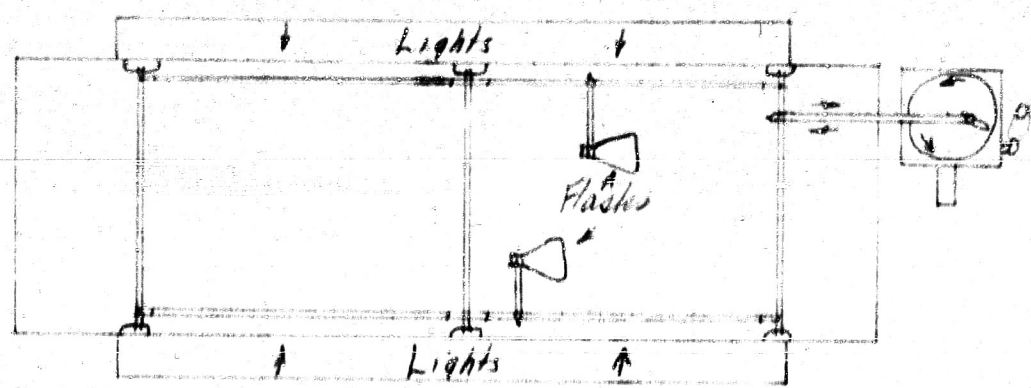
B. Aeration of *G. catanella* cultures

Three aeration methods are being tried at present.

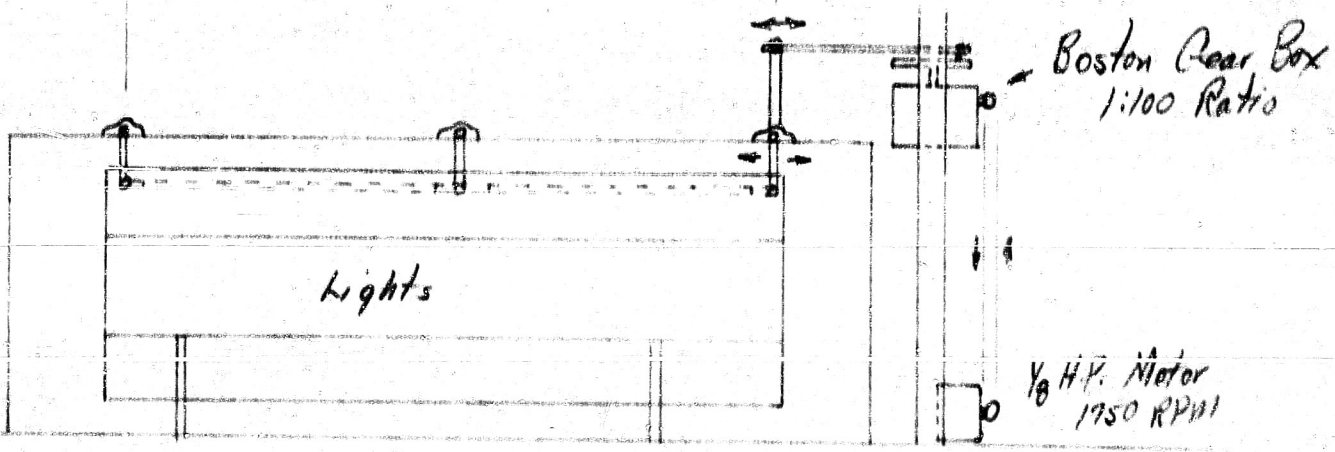
1. Diffusion through cotton plugs in the test tube cultures.
2. Bubbling air into the 20 liter culture bottles.
3. Gentle agitation of the cultures in Erlenmeyer flasks by the use of a shaking apparatus.

The following is a description of the apparatus which has been attached to the tank described in the Final Report on Contract No. W-18-064-CN-251 (15 June 1949 to 15 April 1951).

Top View



46"



Side View

Preliminary work indicates that the cultures aerated by agitation give a better yield of poison than any other method yet tried, but still not as much as desired. Further work is being done.

C. Culture Media

Most of the culture work done during the period covered by this report was done with Media No. 6 as described in previous reports. Recently Media No. 8, an artificial sea water, also described in a previous report, has been tested, but the work has not sufficiently advanced to permit a report at this time.

If artificial sea water can be made which is as good as the fresh natural sea water, then one of the unknown factors in this problem would be eliminated.

The use of a soil extract as a supplement to any culture media is still a necessity and various methods of extraction of the soil have been tried.

1. Heat extracted and heat sterilized
2. Heat extracted and filter sterilized
3. Shake to extract and heat sterilized
4. Shake to extract and filter sterilized

Preliminary tests indicate that media containing soil extract prepared by Method 3 yields a more consistent potency of poison. These extracts are still being evaluated.

A change has been made in the preparation of any culture media. The various components such as sea water, soil extract, and salts are sterilized separately and then combined to make the complete media. This has eliminated the precipitate that was encountered whenever all the ingredients were autoclaved together.

Current plans include a titration of the various ingredients currently being added as supplements in Media No. 6. After this has been done, various organic compounds will be tried as supplements. The first attempts will be with mixtures of amino acids and vitamins.

We also plan to de-ionize the best obtainable media and then reintroduce the various inorganic constituents in an effort to evaluate the effects of certain inorganic elements such as iron, calcium, lead, etc. All of this will be done concurrently with attempts to rid the culture of bacteria.

D. Bacteria

Several attempts have been made to eliminate bacteria from the culture by various methods.

Merthiolate and streptomycin were tried, and it was found that any concentration sufficient to kill the bacteria was also lethal for the Gonyaulax. A few attempts were made to wash the Gonyaulax free of bacteria; this too was unsuccessful. More attempts will be made, because without a bacteria-free culture the nutritional requirements of the Gonyaulax cannot be studied. Furthermore, it is not known if the Gonyaulax require bacteria or their end products.

During the attempts to rid the culture of bacteria, the types of bacteria have been observed. There are usually four distinct colony types seen in 48 hr. cultures on sea water agar at room temperature.

	Colony appearance	Gram Stain Reaction
A	Large (3 mm), very hard white, crenated.	Very difficult to smear. Slender, faintly staining rod. Possibly G pos.
B	Smaller (2 mm), white center, clear edge, soft	Gram neg. pleomorphic rod. No spores seen.
C	Same size as B, white, mucoid.	Gram neg. rod with many spores.
D	Very small, white, opalescent, soft.	Repeated attempts failed to yield smear that would take Gram stain.

Generally one or two of the above types predominate in the *G. catanella* cultures. Usually types B and C or B and D.

Crude bacterial counts from the 20 liter cultures show from 50,000 to 100,000 bacteria per ml. at the end of the 10-14 days incubation period at 14-16°C. No visible cloudiness of the cultures is observed. Counts were made on sea water broth medium to which 1.5% Bacto-agar has been added.

Further attempts will be made to obtain a bacteria-free culture by the use of antibiotics.

E. Harvesting the Culture

A variety of methods have been tried to find the method that will yield the highest percentage of poison.

The procedure that had been routine in this laboratory to harvest and extract the culture used a Celite layer as a filter, then

acidified 50% Ethyl alcohol poured through to extract the poison.

By further extracting this Celite layer with two portions of hot acidified 50% ethyl alcohol, it was found that 150-200 mouse units may be lost by remaining in the Celite. Also the second fraction contained approximately 1.0 M.U./ml. which is considerably below the level detectable by the mouse test method unless the material is evaporated and concentrated.

Other methods of harvesting and extraction have been tried; they include filtration through scintered glass filters, filter paper, filter membranes and porcelain discs.

At present the following method is being used to harvest the 20 liter *G. catanella* cultures. -

The culture is filtered through a micro-porous porcelain filter (Buchner type) porosity 10 with a vacuum sufficient to maintain a steady flow from the filter. 15 liters filtered in 1½-2 hours at this rate. To extract the poison, 350-400 ml. of 50% ethyl alcohol acidified with 1.0 ml. conc. HCl/liter is poured onto the filter and permitted to gravity flow through the *Gonyaulax*. This extraction is done in a refrigerator at + 4°C. and usually allowed to stand 16-18 hours. The alcohol that has not passed through at the end of that time is drawn through with a slight vacuum. The last portion of the alcoholic extract should be clear and colorless. If not, more alcohol is added until the filtrate comes through the filter colorless. Prior to performing the mouse tests, this filtrate is vacuum distilled to approximately one-half the original volume.

The above described method gives the best yield of poison for the amount of manipulation required.

For comparison, 80 or 120 ml. aliquots of the cultures are centrifuged at 2000 RPM for 15 minutes, the supernate is decanted and

the sediment extracted by boiling for 30 minutes in 0.10 N HCl. The poison value obtained serves as a measure of the poison available from each culture.

F. Glassware Washing

Numerous methods have been tried. As previously reported, acid washing followed by 7-8 rinses with distilled water has been the accepted method.

Considering the possibility that the glassware may be the cause of the wide variations noted between counts in consecutive tubes, dichromate cleaning solution has been replaced by a detergent followed by numerous rinses. However, this method of cleaning in no way altered the wide variation in results.

Recently all glassware has been washed only with running tap water followed by 3 rinses with distilled water, and still the variations occur.

Numerous investigators have made the same observation and reported tubes in a series showing good growth while others in the same series show no growth and even death of the inoculum. Thus far no explanation has been offered. We have not observed this total death of a culture when 250 ml. flasks have been used as the culture vessel even though they have been washed in the same manner as the test tubes.

G. Mussel Feeding Experiment

18-24 mussels were gathered from a single mussel bed and brought into the laboratory, where they were cleaned of barnacles, limpets, etc. and kept in sea water which was continuously aerated. Fresh sea water

was added daily. After one week in the laboratory under these conditions, the poison had dropped from 20 MU/mussel to 10 MU/mussel.

At this point 6 mussels were placed in a container and 15 liters of *G. catanella* culture poured over them. Three mussels were removed after 24 hours in the culture and since there were many viable *Gonyaulax* present, 3 other mussels were placed in the culture.

After 72 hours, there were three groups of 3 mussels that had been in the culture 24, 48, and 72 hours respectively. The poison content was determined by the usual method, giving the following results -

	Length of time exposed to culture (Hours)			
	0	24	48	72
MU/ mussel	10	11.1	105	40

At the end of the 72 hour period, 2000 Mouse units were recovered from the organisms remaining in the water in addition to the poison recovered from the mussels. We plan to run this experiment 3-4 more times with mussels as well as clams.

H. Poison Shipments

To determine if there was any loss of poison due to deterioration during shipment, two lots of poison were divided, $\frac{1}{2}$ put on Amberlite IRC-50 and the other $\frac{1}{2}$ shipped as the liquid alcoholic extract.

Assays made at Camp Detrick indicate that shipment in the form of the liquid alcoholic extract show little or no loss in transit. As a result of this, poison obtained from *Gonyaulax* cultures in this laboratory are kept refrigerated until ready for shipment, then shipped in the form of the alcoholic extract.

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The ground mussel liver material was also kept under refrigeration while awaiting shipment. During this time, the pH was periodically checked and maintained below pH 3.0 with conc. HCl.

On 12 August 1952 the mussel liver material collected 23 July, 1952 and 26 July 1952 was shipped to Camp Detrick.

On 1 October 1952 the mussel liver material collected 6 August 1952 and some Gonyaulax culture extract was shipped to Camp Detrick.

Mouse units of poison shipped to Camp Detrick during the period covered by this report -

12 August 1952	2,427,250	(23 July collection)
	1,765,000	(26 July collection)
1 October 1952	1,650,000	(6 August collection)
	<u>78,365</u>	(culture extracts)
Total	5,920,615	Mouse Units

K. F. Meyer

K. F. Meyer, M. D.

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