

**Final Research Report**

**FREEZE CONCENTRATION OF  
TOXIC POLLUTANTS  
FOR BIOASSAY**

by  
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**OWWR No. A-018-ORE**

Toxicity  
Bioassay

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RESEARCH PROJECT COMPLETION  
and  
TERMINATION REPORT  
entitled

FREEZE CONCENTRATION OF TOXIC POLLUTANTS  
FOR BIOASSAY

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## ABSTRACT

A freeze concentration process was investigated as a possible method whereby sub-lethal concentrations of toxic substances in aqueous solution could be quantitatively concentrated for analysis by standard acute toxicity tests. Excellent chemical recoveries were obtained for a sodium chloride control sample, however, two toxic substances, methylmercuric chloride and dieldrin were not concentrated quantitatively. A dilute sample of kraft pulp mill effluent was also freeze-concentrated and evaluated by chemical analysis. Bioassays using newborn guppies were undertaken on unconcentrated and freeze-concentrated solutions of dieldrin. These experiments revealed that the freezing process did not alter the toxic nature of the compound. In general, the results of this study showed that the freeze concentration method is not a feasible approach for concentrating dilute samples for acute toxicity analysis.

## INTRODUCTION

One of the most serious and perplexing problems currently confronting local, state and federal regulatory agencies responsible for protecting the aquatic environment is the establishment and subsequent monitoring of reasonable and safe standards relative to pollutant toxicity. It is important to consider not only types and concentrations of substances which are acutely toxic to organisms, but also those types and concentrations which exert sub-lethal or chronic toxicity.

A common measure of the toxicity of a pollutant is the  $TL_m$ , i.e., median tolerance limit. The  $TL_m$  is determined through a series of standard bioassays (1) and represents the concentration of pollutant which will kill 50 percent of the exposed test organisms in a specified time period. Unfortunately the bioassay procedure commonly used to measure acute toxicity specifies an exposure period of 48 to 96 hours to achieve high sensitivity. Also, since most species of test organisms used in the bioassay tests require dissolved oxygen, some form of aeration is required in the test tanks. Prolonged aeration tends to purge potentially toxic gases and other volatile substances from a polluted sample. Furthermore, aeration provides mixing and dissolved oxygen which promotes biological degradation and alteration of many toxic organic substances.

The lethal response of a toxic pollutant is approximately proportional to concentration of toxicant times exposure period, i.e.,  $kill \sim c \times t$ . Therefore, slightly toxic concentrations of pollutants require long exposure periods to kill one or more test organisms. Since long exposure periods generally result in reduction of toxicity in a sample and adaptation by test organisms, a more feasible way to achieve high sensitivity might be to quantitatively concentrate toxic substances and test for shorter exposure periods. Not only would the aforementioned test limitations be circumvented, but test results would be available in a shorter time.

The standard acute bioassay procedure (1) is valid only for wastes which exhibit sufficient toxicity to elicit a death response by test organisms during a 1-4 day exposure period. Warren (2) and Doudoroff (3) recommend that an application factor of about 0.1 be applied to acutely toxic wastes. This means that a waste with sufficient toxicity to kill 50 percent of a group of test organisms should be given a 10-fold dilution prior to discharge to be "safe." If a waste has a toxicity level just below that which causes death, however, no application factor is applied, yet the waste could have harmful, chronic effects on aquatic life.

Present methods for evaluating sub-lethal stresses in fishes and other forms of aquatic life include growth factor, swimming speed, respiratory pattern, egg viability and others. These tests require highly trained professional personnel and are costly and time-consuming to perform; consequently, these methods are seldom used in field situations.

## PURPOSE AND SCOPE

This research investigation was intended to explore and subsequently develop a quantitative technique whereby sub-lethal concentrations of toxicants could be concentrated for direct evaluation by standard bioassay procedures. The freeze concentration technique described by Shapiro (4,5) and Kammerer and Lee (6) was chosen for evaluation in this study.

The research was conducted during a two-year period extending from July 1, 1972 through June 30, 1974. The first phase of the project was devoted primarily to the design, fabrication and preliminary testing of the freeze concentration apparatus. A series of freeze concentration experiments were performed during the second year to determine the overall feasibility and reliability of this technique for concentrating toxic substances. A third year of research was suggested in the original proposal; however, the marginal results obtained during the second year of the study led this investigator to conclude that a third year would probably not prove to be fruitful. Consequently, the third year of funding was not requested.

## EXPERIMENTAL

### Methods and Materials

#### Sample concentration

Several methods of sample concentration were considered for this application, including vacuum distillation, solvent extraction, electrodialysis, reverse osmosis and freeze concentration. With the exception of freeze concentration, all methods had numerous practical limitations such as loss of volatile substances and dissolved gases, alteration of the chemical nature of substances, incomplete recovery of dissolved substances and difficulty of operation.

The process of freeze concentration was studied as early as 1944 (5). Since that time numerous investigators have used the process for concentrating organic substances (7,8,9,6), volatile chemicals (5,10), biologically-active materials (11,12), minerals (13,14), and dilute solutions (15,16). Kammerer and Lee (6) reported in 1969 on a radioactive tracer study of the freeze concentration process. They found that the process provided nearly 100 percent recovery of soluble substances including volatile chemicals. Furthermore, they found that freezing did not alter the chemical nature or function of the concentrated substances. The freeze concentration process is relatively simple and inexpensive to operate. Samples ranging in volume from one to one hundred gallons or more can be concentrated 50-fold or higher.



### Freeze concentration apparatus

A freeze concentration apparatus similar to that described by Shapiro (4) was fabricated for this study. The essential components of this apparatus, shown in Figure 1, are a condensing unit powered by a 0.5-hp compressor and a 55-gallon (208 liters) stainless steel drum. The stainless steel drum provided a clean, durable and corrosion resistant container for sample concentration. Copper tubing was coiled around the exterior of the drum and welded in place with an acetylene torch and bronze rod. This was a long and tedious task since careful bending of the tubing was required to insure a close fit to the tank and good heat transfer. Care was also necessary to avoid cracking the tubing or the tank with concentrated heat application from the acetylene torch. Freon-12 was circulated through the coils and provided a freezing capability of  $-30^{\circ}$  F. A thermostat, with a sensor positioned inside the drum, provided a positive control on freezing temperature and rate of freezing.

Plastic drain fixtures similar to those described by Kammerer and Lee (6) were originally installed in the bottom of the stainless steel drum. Subsequent toxicity studies showed, however, that the plastic tended to adsorb or chemically react with some of the toxic substances in the drum. Consequently, it became necessary to replace the plastic fixtures with stainless steel fixtures. A 1/8-hp propeller stirrer was mounted on the housing as shown in Figure 1, and was operated at 1750 rpm during test runs.

Tests and analyses.

Standard bioassays on collected samples were performed by personnel from the OSU Department of Fisheries and Wildlife at the Oak Creek fisheries laboratory. Newborn guppies were chosen as the test organisms for this study because of their small size, availability, and wide usage as test organisms by other investigation. All water used for controls or sample dilution was obtained directly from Oak Creek.

Numerous chemical analyses were also required to quantitatively determine the percent recovery of components in samples undergoing freeze concentration. The following analyses were undertaken during this study by the listed procedure.

<u>Chemical (or Parameter)</u>	<u>Method of Analysis</u>
SO <sub>4</sub>	Standard Methods (1)
Cl <sup>-</sup>	Standard Methods (1)
COD (in kraft effluent)	Standard Methods (1)
Na <sup>+</sup> , Ca <sup>++</sup> , K <sup>+</sup> , Fe <sup>++</sup> , Fe <sup>+++</sup> (in kraft effluent)	atomic spectrographic analyses (17)
dieldrin	electron capture gas chromatography (18)
methyl mercuric chloride	flameless atomic adsorption spectrophotometer (19)

### Concentration procedure.

Since relatively large quantities of concentrated samples, 30 to 60 liters, were needed for subsequent bioassays, an original sample volume of 166 liters (43 gallons) was used throughout the experimentation. Larger samples were not feasible in the 55-gallon drum because of liquid losses induced by stirring during the initial phases of the freezing operation. The stirring propeller was maintained at an 8-inch (20-cm) immersion depth throughout the freezing operation to insure adequate mixing without significant splashing.

During the freezing operation, an ice layer developed along the side walls and increased in thickness toward the center. At the completion of the run, a thick ice cake was formed around a central core of concentrated solution. This concentrate was then withdrawn through a stainless steel valve at the bottom of the drum for subsequent chemical and/or biological analyses.

## RESULTS

### Oak Creek water control.

The first experiment was undertaken to determine whether the raw water from Oak Creek (which was used for the bioassay controls and sample dilution) could be concentrated 3- to 5-fold without evidence of toxicity. Raw creek water samples were run in duplicate, one concentrated by a factor of 3.57 and the other by a factor of 3.61. Since the concentrated samples showed visible signs of gas supersaturation, the samples were allowed to degasify at ambient conditions for 2 days prior to biological testing. Both concentrated samples were found to yield 100 percent survival of the test fishes, indicating the apparent absence of acute toxicity in the samples.

### Chemical recovery.

Experiments were next performed to measure the percent recovery of components in a sample which was freeze concentrated. The chloride ion ( $\text{Cl}^-$  from  $\text{NaCl}$ ) was selected as a test chemical because of its high water solubility and ease of measurement. Recovery of compounds, expressed as percent recovery, was calculated from the following expression:

$$\% \text{ recovery} = \frac{(\text{final concentration})}{(\text{initial concentration})} \div \frac{(\text{initial volume})}{(\text{final volume})} \times 100$$

The first test run yielded a chloride recovery of 86.8%, i.e., of the total amount of chloride added to the unconcentrated sample, 86.8% was recovered in the core of concentrate after a 2-day freezing period. During this initial run, the mixer propeller was submerged only 4 inches (10 cm) into the liquid in the drum. This resulted in considerable splashing and physical loss of sample. Also the splashing action allowed the concentrated portion of the sample to "puddle" on the frozen ice cake and salt crystals could be seen on the top of the ice. Splashing was nearly eliminated in subsequent runs by maintaining an 8-inch (20 cm) propeller submergence at all times during the freezing operation.

The next two runs with the chloride ion yield recoveries of 96.4% and 95.0% with liquid volume concentrations of 2.64 and 3.19 respectively.

These results suggested to the investigators that good chemical recoveries were possible, at least with highly soluble ionic substances.

Experimentation was then undertaken with two toxicants which were to be used in subsequent bioassay experiments. These were dieldrin, a chlorinated hydrocarbon commonly used as a pesticide, and methylmercuric chloride, a heavy metal derivative that has been the subject of considerable study in recent years.

Recoveries of these substances were quite poor as shown by the data in Table A below:

Table A

Percent Recovery of Dieldrin and Methylmercuric Chloride  
after Freeze Concentration

<u>Toxicant</u>	<u>initial conc. ug/l</u>	<u>final conc. ug/l</u>	<u>liquid volume conc. factor</u>	<u>percent recovery, %</u>
1) Dieldrin	1.8	2.7	2.83	53.0
2) Dieldrin	5.7	16.5	5.04	57.4
3) Dieldrin	8.5	24.6	3.89	74.4
4) Methylmercuric- chloride	8.0	24.0	5.07	59.2
5) Methylmercuric- chloride	32.5	82.5	5.23	48.6
6) Methylmercuric- chloride	75.0	75.0	4.07	24.5
7) Methylmercuric- chloride	30.0	49.0	6.92	23.6

The poor recoveries of these two compounds might be attributed to four major types of losses: entrapment in the matrix of the

of the ice formed during concentration; volatile losses to the atmosphere; direct losses from samples as the compounds adhered to the sides of sampling containers; and experimental error in the analytical determinations of the toxicant concentrations.

In order to determine the extent of losses in the ice matrix the concentration of toxicants in the ice were measured. This was accomplished in the following manner. First all core concentrate was withdrawn from the drum. The inner surfaces of the ice layers were then rinsed with distilled water. Next the ice was allowed to melt and the melted liquid was withdrawn and tested.

The dieldrin concentration in the ice composite from run #2 was 2.3 mg/l, representing 32.3% of the initial quantity of dieldrin in the sample. The ice composite from run #4 yielded a methylmercuric chloride concentration of 2.25 mg/l, representing 22.6% of the initial quantity of that compound in the original solution. These must be considered as very significant losses of sample components.

Several previous researchers have practiced "ice washing" at the end of each concentration run. This was not practiced during the majority of this study for the following reasons:

- (1) High recoveries of chloride ion were obtained without washing in preliminary runs.
- (2) Previous researchers who practiced ice washing worked with a small rotating flask. The ratio of surface area of ice to final volume of concentrated solution

in those studies was far higher than that found in this study. The fraction of solute trapped in the film of water adhering to the ice cake, which ranged from 2-10% in previous studies, would be insignificant with respect to the losses encountered in this study.

- (3) Rapid mixing of the contents of the freezing drum was continued during sample withdrawal, which would tend to keep "film losses" to a minimum. This continued mixing was not practiced during previous studies.

Due to the poor recoveries obtained with dieldrin and methylmercuric chloride, another run was made to determine whether ice washing might restore a significant portion of the chemical losses. At the completion of run #7, the surface of the ice was "washed" with distilled water. Laboratory analysis revealed that the amount of methylmercuric chloride recovered in this manner represented less than 0.2% of the amount of the compound in the original sample. This observation substantiated the theory that film losses are minimal with this apparatus.

The total quantities of dieldrin and methylmercuric chloride in the combination of the concentrated liquid phases and the ice phases in runs #2 and #4 represented 89.7% and 81.8% respectively of the initial quantities of those compounds added to the original samples.

Since methylmercuric chloride is considerably more volatile than dieldrin it would be expected that losses through evaporation to the atmosphere would be greater for methylmercuric chloride



than for dieldrin. Although no quantitative data were collected to substantiate this hypothesis, losses of methylmercuric chloride appeared to be greater than the losses of dieldrin.

Both of the compounds tested are noted for their propensity to adhere to glassware. With the low concentrations used in this study, losses of these compounds on the sides of the sample containers could be a significant source of error. If more highly concentrated solutions had been used, the resultant error would have been less significant on a percentage basis.

Analytical errors in measurement should be minimal for both compounds tested. Methylmercuric chloride analyses were performed in duplicate by the flameless atomic absorption procedure. Mercury concentrations obtained from this procedure are accurate to 0.1 ug/l for concentrations of 1-10 ug/l and to 1.0 ug/l for concentrations above 10 ug/l. Dieldrin analyses, performed with electron capture gas chromatography are accurate to 0.1 ug/l in the concentration ranges tested. Errors of these magnitudes do not appear to be significant with respect to the range of values obtained in this study.

## Bioassays

Standard 96-hour bioassays with newborn guppies were performed on the freeze concentrated samples from runs #1 and #3 (noted in Table A) to determine whether the process of freeze concentration altered the toxicity of dieldrin. The following results were obtained:

### Run #1

TL<sub>m</sub> unconcentrated sample = 1.0 ug/l

TL<sub>m</sub> concentrated sample = 1.8 ug/l

### Run #3

TL<sub>m</sub> unconcentrated sample = 3.5 ug/l

TL<sub>m</sub> concentrated sample = 4.0 ug/l

The TL<sub>m</sub> of dieldrin for newborn guppies is reported in the literature to be 3-5 ug/l.

The fisheries specialist who performed the bioassays reported that the analytical results from tests performed for the first bioassay test were possibly in error since the TL<sub>m</sub>'s found in both the freeze concentrated sample and the blank sample were approximately half of the reported TL<sub>m</sub> values for dieldrin. The results of the first test were therefore suspect.

The second bioassay test with dieldrin indicated that the difference in TL<sub>m</sub> between the frozen and unfrozen samples of dieldrin was not significant. Consequently, it could be assumed that the freezing process did not significantly alter

the toxic character of the compound.

No bioassay results are reported for freeze concentrated methylmercuric chloride because of the very poor recoveries found in the testing program.

Freeze concentration of kraft mill effluent

After completing the series of experimental runs with dieldren and methylmercuric chloride and noting the very poor and erratic chemical recoveries, the investigators were pessimistic about the likelihood of successful use of this apparatus as a tool for use in biological toxicity testing. A final run was undertaken, however, with a dilute sample of kraft pulp mill effluent. Recoveries of several ions (or parameters) were determined as shown below: (Liquid volume concentration = 5.93)

<u>ion (or parameter)</u>	<u>% Recovery</u>
*SO <sub>4</sub>	81
*COD	75
**Na <sup>+</sup>	86
**Ca <sup>+</sup>	72
**Mg <sup>++</sup>	90
**K <sup>+</sup>	107
**Fe <sup>++</sup> & Fe <sup>+++</sup>	108

\* Standard Methods testing procedures (1)

\*\* Atomic spectrographic analysis (17)

If the recoveries of various ions had been uniform, either high or low, future testing would have been reconsidered to determine which parameters such as pH, mixing speed, rate of freezing, etc., might be adjusted to enhance recoveries. The

erratic nature of the results indicated, however, that percent recovery for freeze concentrated substance varies widely and suggested that no single correction factor could be applied to adjust results.

## DISCUSSION

Based upon the reported work of Shapiro (4,5) and Kammerer and Lee (6) with freeze concentration of organic and inorganic substances, this investigator was confident that the freeze concentration approach would prove successful in concentrating samples containing toxic substances. Unfortunately, results of this study revealed that some substances apparently are not readily concentrated quantitatively. Since the reliability of toxicity information would depend heavily upon uniform concentration of all substances in a sample, it was concluded that this approach is not a feasible method of determining sub-lethal toxicity.

Since excellent recoveries were obtained with chloride samples, it was concluded that the apparatus and procedures followed were acceptable and similar to that described in the literature. No references were found in the literature regarding the recovery of dieldrin or methylmercuric chloride after freeze concentration. Perhaps the chemical properties of these compounds differ significantly from the humic acids studied by Shapiro (4) and volatile organics studied by Kammerer and Lee (6) and resist uniform concentration by freezing.

Furthermore, it is important to point out that the sample volumes used by the aforementioned investigators were considerably smaller than those needed for bioassay measurements. There

is no readily explainable reason, however, why sample size should affect chemical recovery.

It became readily apparent after several experimental runs with dieldrin and methylmercuric chloride that the objectives of this research could not be realized. Consequently, this investigator elected to withdraw his original request for a third year of research support. Even though the results of this study might be considered to be negative, a substantial amount of worthwhile information was obtained. Furthermore, two graduate students were provided an excellent opportunity to search for answers to a number of complex and perplexing problems.

## CONCLUSIONS

Based upon the results obtained during this study the following conclusions are presented:

1. Sodium chloride solutions can be quantitatively freeze concentrated.
2. Selected organic substances, specifically dieldrin and methylmercuric chloride, cannot be quantitatively freeze concentrated in large volumes.
3. Freeze concentration does not appear to be a feasible sample preparation technique for biological analysis of sub-lethal concentrations of toxic substances.



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19. Methylmercuric Chloride Analysis. Conducted by the Fisheries and Wildlife analytical laboratory at Oregon State University.

## PERSONNEL

Personnel actively involved with this research study included:

Frank D. Schaumburg, Ph.D., Principal Investigator

Michael R. Harris, Sanitary Engineer, Graduate Research  
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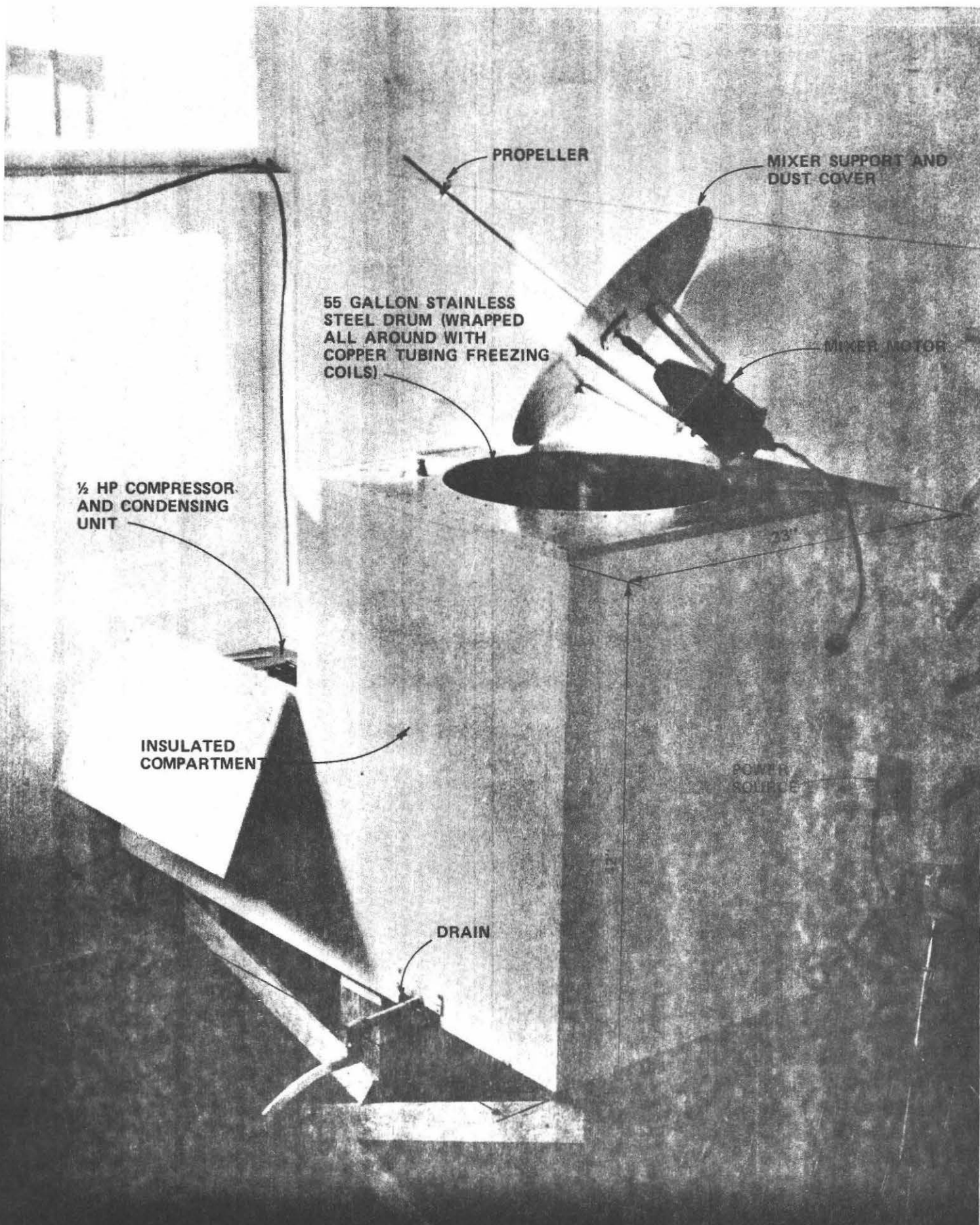
Mark Lasswell, Research Assistant

Wayne Seim, Fisheries Specialist and Advisor

## PUBLICATIONS

One publication resulted from this research study.

Master's Project Report by Michael R. Harris entitled  
"Freeze Concentration of Toxic Pollutants for Bioassay."  
May, 1974



PROPELLER

MIXER SUPPORT AND  
DUST COVER

55 GALLON STAINLESS  
STEEL DRUM (WRAPPED  
ALL AROUND WITH  
COPPER TUBING FREEZING  
COILS)

MIXER MOTOR

1/2 HP COMPRESSOR  
AND CONDENSING  
UNIT

INSULATED  
COMPARTMENT

DRAIN

POWER  
SOURCE

FREEZE CONCENTRATION TEST UNIT