THE MULTIPLE TOXICITY OF VANADIUM, NICKEL, AND PHENOL TO FISH

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ABSTRACT

A preliminary study of the toxicity of vanadium, nickel, and phenol to juvenile rainbow trout, in pure solutions and in certain mixtures, has been conducted.

In lethal tests, the 96 h LC_{50} for vanadium, nickel, and phenol was 11.5, 28, and 10 mg/L, respectively. Vanadium solutions caused the death of test organisms throughout a 14 d period, at which time the experiments were terminated. No incipient lethal level (ILL) was evident within the time period studied, a pattern which is often evoked by toxicants that accumulate. Similarly, there was no apparent ILL for nickel in a toxicity test which lasted 10 d. In contrast, the ILL for phenol was correlated with a time of approximately 22 h.

The extensive damage to the secondary lamellae of gills observed in fish exposed to vanadium was dose-related. This correlation suggested that gills are a critical site for the lethal action of vanadium. Histopathological studies were limited to vanadium-exposed fish.

In sublethal tests of nickel, effects on various properties of blood were investigated. At the lowest concentration of nickel, 0.8 mg/L, only the level of serum proteins was affected significantly. No impairment to osmo- or iono-regulatory capacity was noted in test fish submitted to a saline stress following exposure to sublethal levels of nickel in freshwater.

In multiple toxicity tests, it was concluded that the lethal potency of vanadium and nickel in certain binary mixtures is less than strictly additive. This "antagonistic" pattern of multiple toxicity progressively decreases through a range of binary solutions in which the concentration of vanadium relative to the total concentration increases. Where vanadium greatly predominated in mixtures, about 75% of the total effective concentration, the toxicity of the two metals was strictly additive.

Vanadium and phenol were found to act independently as constituents of lethal, binary mixtures. Only when a constituent's concentration was above its respective toxicity-threshold would that constituent contribute to the joint potency of vanadium-phenol mixtures. This latter pattern of multiple toxicity is designated response addition.

Synergism of a form designated as supra-additive was observed in lethal tests of tertiary mixtures which contained vanadium, nickel, and phenol. The degree of synergism increased through a series of mixtures in which the ratio of the concentration of phenol to that of the total mixture decreased. This synergistic phenomenon reached a plateau at a magnitude of effect which approximately doubled that predicted. The significance of these results is discussed in reference to further research requirements and the setting of water quality standards to safeguard aquatic organisms.

A dry-ash procedure for the precise determination of vanadium and nickel in fish tissue was developed. The vanadium and nickel content of whole tissue of rainbow trout with no known previous exposure was 1.5 and 0.3 μg/g (dry weight), respectively.

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1. INTRODUCTION

1.1 PRIMARY OBJECTIVE

The primary objective of this study was to provide a preliminary assessment of the toxicity of vanadium, nickel, and phenol mixtures to fish.

An industrial program as ambitious and complex as the mining and processing of the bitumen in the Athabasca Oil Sands raises the possibility of introducing an array of chemical substances into aquatic ecosystems. These substances may arise from various point and non-point sources; e.g., industrial waste pipes, effluent containments, open-pit mines, and stack emissions. Their chance convergence in natural water bodies creates mixtures. Because these mixtures happen in situ, it is likely that the species and concentration of the individual chemical constituents vary between locations in receiving waters. Singly, each chemical pollutant may be at a level which is harmless; in combination, the same substances may have a detrimental effect on the indigenous biota. Such toxicities which are uniquely attributable to mixtures of substances are categorically identified by the term, multiple toxicity.

The three chemical substances examined in this multiple toxicity study were selected because of the following considerations:

1. Vanadium and nickel are the two major trace metals associated with petroleum crude and related products (Jacks 1976); and
2. Phenol is the primary member of various phenolic compounds which collectively are amongst the most prevalent and toxic organic substances discharged in liquid wastes by the petroleum industry (Côté 1976).

There have been a few reports in the literature that allude to multiple toxicity problems involving one or more of the three selected substances. Doudoroff and Katz (1953) have described multiple toxicities associated with certain heavy metal mixtures containing nickel. An epidemiological study has demonstrated a correlation between the metals vanadium and nickel, and the incidence of chronic vascular diseases in man (Hickey et al. 1967). Brown et al. (1969) reported that the potency of mixtures containing phenol and the heavy metal copper was greater than expected following the premise that each constituent operates discretely within an organism. This evidence, although limited, creates concern that vanadium, nickel, and phenol may interact physiologically to harm organisms exposed to their mixtures.

1.2 ADJUVANT PROJECTS

Certain exploratory and preparatory projects for the study of multiple toxicity at sublethal levels were carried out as follows:

1. Time-dependent, toxicity curves for each discrete pollutant were developed for the purpose of establishing their respective incipient lethal levels, a standard criterion that distinguishes sublethal from lethal stresses;
2. A preliminary, histopathological investigation was conducted in search of the critical target tissue(s) and organ(s) that is/are damaged by vanadium within test fish. Evidence of lesions could provide a guide for future research;
3. The osmoregulatory functions of test organisms were explored as a sensitive physiological system for detecting the sublethal effects of nickel. This system was selected because heavy metals have been shown to interfere with ion transport across gill epithelium (Lewis and Lewis 1971; Waldichuk 1974),

and because osmo- and iono-regulation impose(s) the largest expenditure of energy within the standard metabolism of fish (Renfro et al. 1973). Furthermore, in the mining of the Athabasca Oil Sands, salt deposits have been encountered with ensuing release of saline effluence into surface waters. A subsequent increase in salinity may present an additional and significant stress on the osmo- and/or iono-regulatory functions of indigenous, aquatic biota; and

4. Precise techniques for the "dry ash" analyses of each metal, vanadium and nickel, in fish tissues were developed. "Acid-digestion" procedures which are commonly recommended for tissue analyses of metals have proven unsatisfactory in previous studies in the laboratory.

1.3 GENERAL BACKGROUND INFORMATION ON POLLUTANTS

1.3.1 Vanadium

Levels of vanadium as high as 1600 ppm are found in certain crudes (Bergtsson and Tyler 1976). Within crude oils, vanadium is normally bound to porphyrins (Yen 1972). During the fractionation of crude oils, vanadium complexes may be oxidized and subsequently emitted into the atmosphere as part of a refinery's fly ash particulates (Linton et al. 1976). Nevertheless, significant amounts of the vanadium, which originally occurs in crude oils, remains in fuel oil products. Combustion of these products in industrial and home furnaces provides another airborne source of environmental contamination by vanadium (Jacks 1976). Certain bacteria are known to oxidize vanadium (Zajic 1969) and may represent a means of promoting the leaching of vanadium from mine sites.

Vanadium enters aquatic ecosystems primarily via fallout of fly ash aerosols or raindrops (Tuller and Suffet 1975). The latter authors suggest that pentavalent vanadium is the major

oxidized form which enters natural waters. Deltombe et al. (1966) concluded that $H_3V_2O_7$ is the predominant species of vanadium in waters of pH ranging from 7.0 to 8.0. Vanadium is readily chelated by humic acids in aquatic media (Szalay and Szilagyi 1967). This trait may be a reason for the high proportion of vanadium contamination that accumulates in bottom sediments (Jacks 1976). Levels of vanadium above 2 µg/L in natural waters are thought to be indicative of pollution (Bergtsson and Tyler 1976).

Trace levels of vanadium are essential for the metabolism of many higher organisms (Freiden 1972). A report prepared by the National Academy of Sciences (1974) concluded that prevailing levels of anthropogenic vanadium in the environment do not represent a serious health hazard to man. Sprague et al. (1978) suggested that, amongst heavy metals, vanadium is a moderate toxicant to fish and that 0.17 mg/L represents a reasonable estimate of that level below which most native fishes would not be harmed. However, the capacity of vanadium to interact physiologically with other chemicals to cause detrimental effects in organisms has not been sufficiently studied.

1.3.2 Nickel

An unexplained correlation exists between levels of vanadium and nickel in many crude oils. However, in most of the world's crude oils, the upper limits of nickel's concentration rarely exceed 100 ppm (Milner 1963).

Distillation processes during oil refining tend to concentrate nickel in fuel oils (Andersson and Grennfelt 1973) and eventually this nickel may be released as an airborne contaminant in the combustion of petroleum products (Jacks 1976). Another possible source of nickel that may contaminate the environment is in liquid wastes discharged by petroleum refineries (British Columbia Petroleum Association 1972).

The Environmental Protection Agency's (EPA) red book suggests that nickel levels of 100 µg/L may adversely affect certain freshwater organisms. However, Biesinger and Christensen (1972) found that levels as low as 30 µg/L significantly impaired reproduction in copepods. In comparison, levels of nickel in pure solutions that were less than 0.38 mg/L did not harm the survival, growth, and reproductive capacity of the fathead minnow (Pickering 1974). In studies of mixtures, low levels of this element in combination with certain other heavy metals may have synergistic effects (Hutchinson 1973; Weinstein and Anderson 1978).

1.3.3 Phenol

Phenol and other phenolics are rarely found in crude oil; but, in the refining of the latter, multi-cyclic aromatics (e.g., anthracene and phenanthrene) are converted to monohydric phenols. Production of phenolic compounds may occur in mid-distillation processes such as thermal and catalytic cracking, fractionation, and caustic treatments (Jones 1973; Côté 1976). In addition, phenol is used as a solvent in certain refinery processes (Jones 1973).

Phenolic compounds common to refinery effluents include pure phenol, cresols, and zylenols. The gross production of total phenols that contaminate waste water emissions is typically 72.6 kg/day for a 30 000 B/D refinery (Beychok 1967). Of these, pure phenol represents the largest phenolic component and is considered to be the most potent fish toxicant (Herbert 1962). A strong correlation has been shown between the short-term potency of petroleum effluents and their content of phenol (Côté 1976; Graham and Dorris 1968). EIFAC (1973) has recommended, in criteria to safeguard aquatic organisms, that the levels of monohydric phenols, as discrete agents in freshwaters, not exceed 0.5 to 1.0 µg/L.

2. RATIONALE FOR AN EMPIRICAL APPROACH TO THE STUDY OF MULTIPLE TOXICITY

The experimental approach applied in the following lethal response studies is based on theoretical considerations of multiple toxicity that were originally proposed for quantal data by Bliss (1939) and Plackett and Hewlett (1952). In essence, they derived quantitative formulations for predicting the toxic effects of mixtures that are the consequence of poisonous constituents which act in an organism either similarily, at the same target site, or independently, at different target sites. In this study no attempt was made to discover the actual modes of multiple toxicity. Rather, the empirical data were examined statistically to determine whether they fitted either Bliss's or Plackett and Hewlett's formulations. The mode of multiple toxicity for those models that data were shown to fit was thereupon assumed.

The literature is replete with a confounding array of terms used to identify patterns of response for mixtures of toxicants that are either equal to, less than, or greater than the responses predicted by various hypothetical models. In this study, a terminology has been adopted that is intended to maintain a coherency and avoid the connotation that the mode of action of a toxicant or combination of toxicants is known. The multiple toxicity models and related terminology are described in the following four sections.

2.1 RESPONSE ADDITION MODEL

The model of response addition is based on the postulate that individual toxicants within a mixture may affect different physiological sites and yet contribute to a common response. For example, a hepatotoxicant and a neurotoxicant cause dysfunction in the liver and in the central nervous system, respectively, each of which can lead to the death of an organism. Such toxicants are assumed to be "independently acting". This model infers that

an independently acting toxicant within a mixture contributes to a common response at the level of the whole organism only if its concentration exceeds a threshold level. This threshold is determined empirically through tests which study each toxic constituent singly. Furthermore, this model is applicable only in cases where the presence of one constituent of the mixture does not interfere with the toxicity of any other constituent in the mixture.

According to the model of response addition, the lethal toxicity of a mixture may be predicted from a knowledge of the lethal toxicity of the separate constituents. In this context, the combined lethal potency of the toxic mixture is expressed in terms of the proportions of individuals within a test lot that respond accordingly.

This model, in predicting the toxicity of mixtures, can be adjusted for the fact that, within any population or sample of organisms, the tolerances of individuals to different independently acting toxicants may or may not be correlated. If, for a particular sample of organisms, there is no correlation of tolerances to two response-additive toxicants, A and B, then the proportion of animals which will respond to a binary mixture is predicted by:

$$P_{AB} = 1-(1-P_a)(1-P_b) \qquad (1)$$

where, P_{AB} = proportion responding to mixture of A and B.

P_A = proportion responding to A alone.

P_B = proportion responding to B alone.

When the correlation in tolerances is completely positive, i.e., those animals which are most tolerant to A are also most tolerant to B, the response in a mixture is evoked by the more potent constituent. In this instance the multiple toxicity is given by:

$$P_{AB} = P_A \qquad (2)$$

where, P_A is the greater potency.

If the correlation in tolerances is completely negative, i.e., those animals most tolerant to A are least tolerant to B, and vice versa, then the proportion of animals which will respond in a mixture is predicted by:

$$P_{AB} = P_A (1-P_B) + P_B (1-P_A) \quad (3)$$

The manner in which this model was applied herein is described in Section 3.4.6.4.

2.2 STRICT ADDITION MODEL

The model of strict addition is based on the postulate that individual toxicants within a mixture can affect the same critical organ site. For example, two different trace metals may each bind with sulphydryl groups in gill epithelium to cause protein denaturation that may lead to respiratory dysfunction and eventual death. Such toxicants are described as "similarly acting". The number of sites that are occupied is cumulative for all similarly acting poisons in a mixture. Therefore, in contrast to response additive toxicants, strictly additive constituents of a mixture do contribute to the overall potency of the mixture even if their respective concentrations are below the threshold levels determined for pure solutions.

Bliss (1939) argued that tolerances to similarly acting toxicants are normally and identically distributed within a population of organisms. Therefore, quantal (all or none) response curves for pure solutions of toxicants should be parallel as the slopes are a reflection of the tolerance distribution. However, Plackett and Hewlett (1952) suggested that parallelism was not an absolute indicator of similarly acting toxicants. Consequently, this mode of toxicity was tested herein whether or not the dose-response curves for the discrete toxicants were parallel.

According to the model of strict addition, the lethal toxicity of a mixture to elicit a given response may be predicted from a knowledge of the lethal toxicity of each constituent. In practice, the model of strict addition is tested by first

determining a "relative potency" factor which denotes the ratio between equipotent concentrations of each constituent. This factor is then used to convert the level of one toxicant to an equipotent concentration of the other (see Section 3.4.6.2).

If the respective dose-response curves for the single toxicants A and B are defined by the maximum likelihood probit regressions as follows:

$$Y_A = a_A + b_A \log_{10} C_A \quad (4)$$

$$Y_B = a_B + b_A \log_{10} C_B \quad (5)$$

then, $Y_{AB} = a_A + b_A \log 10\ C_A + C_B$ (in A units) (6)

where, Y_{AB}, Y_A, Y_B = percentage of organisms responding (in probit units) to their mixture or toxicant A or B respectively.

C_A, C_B = concentration of toxicant A or B, respectively.

a_A, a_B = ordinate intercept of dose-response curve for toxicant A or B, respectively.

b_A = common slope as represented by regression coefficient of dose-response curve of toxicant A.

2.3 MODEL OF SUPRA-ADDITIVE SYNERGISM

Toxicants in mixtures that evoke responses which exceed predictions based on models of response addition and strict addition are defined as supra-additive in their mode of multiple toxicity. Supra-additive synergism may result from interactions with pharmacokinetic processes which determine the actual quantity of toxicant(s) available at target sites, or from pharmacodynamic processes which mediate the observed reactions. Theoretically, this form of multiple toxicity may occur in mixtures whose constituents act similarly on the same type of target sites or independently on different types of target sites; however, a distinction between these possibilities cannot be deduced on the basis of whole organism studies.

Unlike the multiple toxicities of response additive or strict additive poisons, the potency of a mixture of supra-additive toxicants cannot be predicted solely from a knowledge of

the dose-response relationships for the constituents. Thus, supra-additive synergism, which often results in unpredictably high levels of response, must be considered a particularly hazardous form of multiple toxicity. A hypothetical example of a response-parameter (e.g., LC_{50}) recorded for a mixture of supra-additive toxicants is plotted in Figure 1.

2.4 MODEL OF INFRA-ADDITIVE ANTAGONISM

Infra-additive toxicants in mixtures elicit responses which are less than those predicted based on models of response addition and strict addition. Interactions with pharmacokinetic or pharmacodynamic processes presumably account for infra-additive antagonism, which may be induced by mixtures whose constituents act on the same or on different types of receptors. Although the potency of a mixture of infra-additive toxicants cannot be predicted solely on the basis of the dose-response relationships for the constituents, this form of multiple toxicity, which often results in low levels of response, is not considered to be particularly hazardous. A hypothetical example of a response-parameter (e.g., LC_{50}) recorded for a mixture of infra-additive toxicants is illustrated in Figure 1.

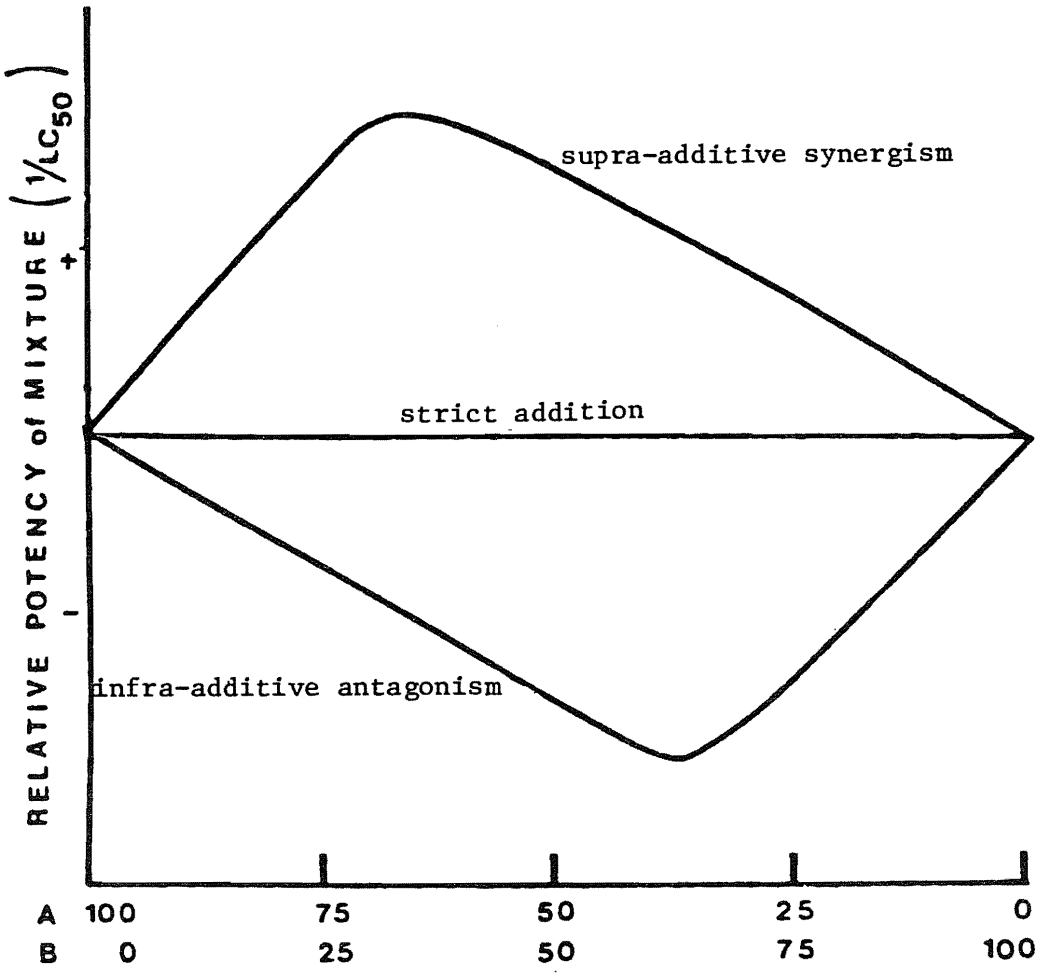

Figure 1. Shown is an isobol representing co-ordinates of equi-response (LC_{50}) for strictly additive constituents, A and B, of binary mixtures in which the relative proportion between toxicants differ. Hypothetical isobols are illustrated for supra- and infra-additivity (i.e., the same magnitude of response is attained at greater and lesser "combined" concentrations, respectively).

3. MATERIALS AND METHODS

3.1 FISH HANDLING

Rainbow trout [*Salmo gairdneri* (Richardson)], of the Donaldson variety (purchased from La Pisciculture Mont Sutton Inc., RR#1, Sutton, Quebec) were allowed to acclimate to laboratory conditions for a period of at least three weeks. During this time, they were held in 500 L tanks through which water flowed at a rate of 5 L/min. Ninety-five percent of the water was renewed every five hours (Sprague 1973). The metabolism of fish was not limited by oxygen availability because monitoring showed that the oxygen content of the water was never less than 80% saturation. This oxygen level was assured by supplying the holding tanks with air stones. Water temperature was maintained at 12 ± 1°C during the acclimation period and throughout all subsequent tests. Thermoregulatory studies in the laboratory have shown that 12°C is close to the preferred ambient temperature of this species.

Selected chemical characteristics of the laboratory's source water are listed in Table 1. Activated charcoal filters were used to dechlorinate the source water delivered to the building through mains from Montreal's drinking water reservoirs. During the acclimation period fish were fed daily ad libitum with Ewos Trout Chow. The feeding times were randomized to prevent fish from being conditioned to a particular feeding-time regime. A daily, 12 h photoperiod of fluorescent light, with an intensity of 538.2 lx at the water surface, was maintained.

3.2 CONTINUOUS FLOW DILUTION APPARATUS

A flow diagram (Figure 2) illustrates the movement of water and toxicant solutions through the toxicity apparatus. Stock solutions of nickel and phenol were held in separate Mariotte bottles which dispensed their contents at controlled rates into a common first-stage dilution chamber. A vanadium solution was metered into the same reservoir from a unique apparatus designed to provide a source of vanadium pentoxide (Section 3.3.1).

Table 1. Chemical characteristics of source water.

Parameter	
dissolved oxygen (% saturation)[a]	85 ± 10 (range)
temperature[a]	12 ± 1°C
pH[a]	7.9± 0.1
alkalinity as ($CaCO_3$)[a]	85 ± 2 mg/L
hardness as ($CaCO_3$)[a]	125 ± 5 mg/L
chlorine[a]	0.02 mg/L
silicate[b]	37.4 mg/L
calcium[b]	37.4 mg/L
magnesium[b]	8.1 mg/L
sulphate[b]	26.0 mg/L
sodium[b]	12.3 mg/L
potassium[b]	1.4 mg/L
fluoride[b]	0.15 mg/L
iron[b]	0.12 mg/L

[a]Analyses performed in lab after charcoal filter.

[a]Analyses performed at the City of Montreal Filtration Plant.

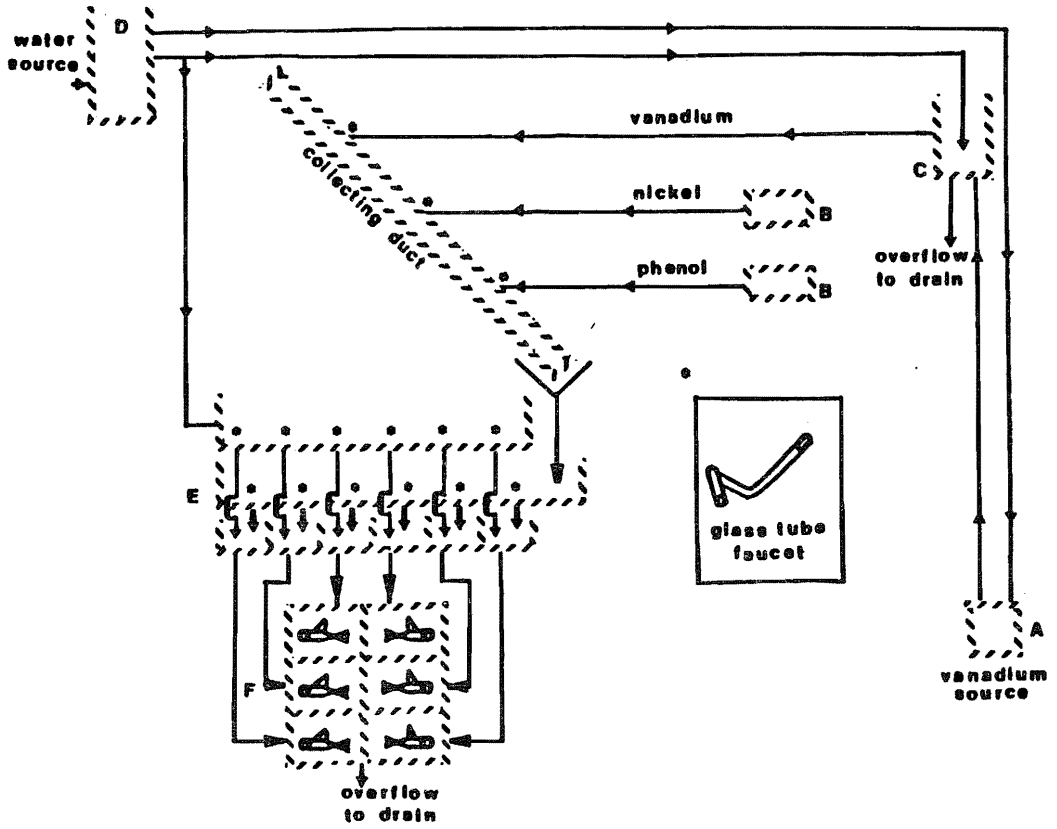

Figure 2. Flow diagram of test apparatus. Diluter apparatus (E) channels solutions of individual toxicants or their mixtures to exposure tanks (F). Shown here are six channels; actual setup provided 10 channels. Flow rate and dilution are controlled by a glass tube faucet located at each asterisk*. (D) is main headbox where water pressure, temperature, and gas content are regulated. (A) represents vanadium source and (C) is a reservoir for first-phase dilution of vanadium. (B) is Mariotte bottle which contains solutions of either nickel or phenol.

Standpipes were employed to govern the heights and thus the volumes of liquid in the upper two chambers of the dilution apparatus. Glass faucets of a design shown in Figure 2 controlled water and toxicant flow. These faucets were inserted through rubber stoppers into their respective chambers. The rate of flow from a faucet is primarily a function of the tube's inner diameter and the hydraulic head which is governed in turn by the vertical distance between the tip of the faucet and the top of a chamber's standpipe. Flow rates can be varied either by changing the inner diameter of a faucet or by altering the height of the hydraulic head. The latter manoeuver is simply achieved by rotating a faucet about its horizontal axis. Each faucet functions independently.

The upper chamber of the diluter is a water head box. A faucet inserted at one side of this head box provides a continuous flow of water into the funnel which also collects stock solutions of one or more of the three toxicants. Consequently, the toxicants are diluted and mixed as they enter the middle chamber. Other faucets located at the front of the diluter pair a series of nine flows of water from the upper head box with a corresponding series of toxicant flows from the middle chamber. As the toxic solutions are collected in funnels, they are combined with water, diluted, and directed to nine of 10 chambers in a series which comprise the lowest reservoir of the apparatus. Tygon tubing delivers the final solutions from their respective, second-phase dilution reservoirs to a corresponding series of exposure tanks. The tenth chamber in the lowest reservoir collects only water which is channeled by tubing to a control tank. Each tank is glass lined and is approximately 68 L in volume.

The apparatus is particularly suited to multiple toxicity studies. Among its advantages are the capacity to provide, within a series, a wide range of precisely determined concentrations, the facility with which the concentrations of toxicants can be changed, the continuous rather than intermittent toxicant flows at any

desired rate, and the versatility in composing mixtures of different proportions as well as different total concentrations. To achieve this latter feature, it is necessary to add first-phase dilution chambers for each of the toxicants. In this arrangement, the discrete stock solutions of each toxicant are initially diluted before being combined during the second-phase dilution step with other constituents of a mixture. In these studies, proportions between contaminants were fixed. This arrangement requires only one first-phase dilution reservoir.

3.3 PREPARATION OF STOCK SOLUTIONS

3.3.1 Vanadium

The most prevalent form of petrogenic vanadium as an airborne contaminant entering natural waters is probably vanadium pentoxide (Tuller and Suffet 1975). Vanadium pentoxide may enter wastewater as particulate matter adhering to fly ash (Linton et al. 1976) or as a dissolved ion (most likely $H_3V_2O_7$) (Deltombe et al. 1966) in raindrops (Jacks 1976). The solubility of vanadium pentoxide in water within the pH range 6.5 to 8.2, which represents most surface water conditions, is low at approximately 0.4 µg/L (Tuller and Suffet 1975). But higher concentrations, up to 46 µg/L, of anthropogenic vanadium have been detected in water courses (Jacks 1976). Thus, it would appear that vanadium can exist in water above its solubility limit as suspended mineral matter (Jacks 1976) or as a complex with organic material such as humic acids (Szalay and Szilagyi 1967).

To simulate, within the limits of the chemical characteristics of the source water, the most likely form(s) of vanadium arising from vanadium pentoxide contamination, a system was devised which delivered vanadium solution from flow-through bottles containing vanadium pentoxide crystals. The vanadium source was prepared by composing an aqueous slurry of vanadium

pentoxide, adding the slurry to six 1L glass jars in which gravel, approximately 1 cm in diameter, had been placed, and allowing the crystals to settle for two days. The gravel served to provide a surface on which the vanadium pentoxide crystals precipitated and to establish a porosity which aided the movement of water through the containers. The flow rate of water which was piped from a head box through the vanadium bottles to the dilution apparatus (Figure 2) was regulated precisely.

The total concentration of the dissolved and particulate vanadium delivered to the dilution apparatus was related directly to the flow rate of water through the vanadium source system (Figure 3). As previously mentioned, this system is designed to provide vanadium in forms which are similar to those that contaminate natural waters. Furthermore, this mode of vanadium delivery does not rely upon acids and bases to solubilize stock solutions. This avoidance is considered advantageous because Na^+ or K^+, as might arise for example from sodium and potassium hydroxide, respectively, enhances the vanadium signal in flame furnace, atomic absorption (A.A.) spectrophotometry (Quickert et al. 1974). This interaction can lead to erroneously high values.

The delivery system provided levels of total vanadium as high as 50 mg/L at the exposure tanks. At this magnitude of concentration, vanadium-contaminated water appeared bright yellow and homogeneous. Neither precipitate nor suspended particulate matter was observed.

Nevertheless, the vanadium source system had some serious operational drawbacks. The concentration of vanadium leaving the vanadium source not only varied with flow rate (Figure 3) but also varied with the passage of time (Figure 4). This feature dictated that vanadium concentrations at C (Figure 2) be assayed at least daily. The information thereby gained was used to compute adjustments to flow rates that were necessary to compensate precisely for any change in vanadium concentration. In practice, it was not possible to assay at this frequency. As an alternative,

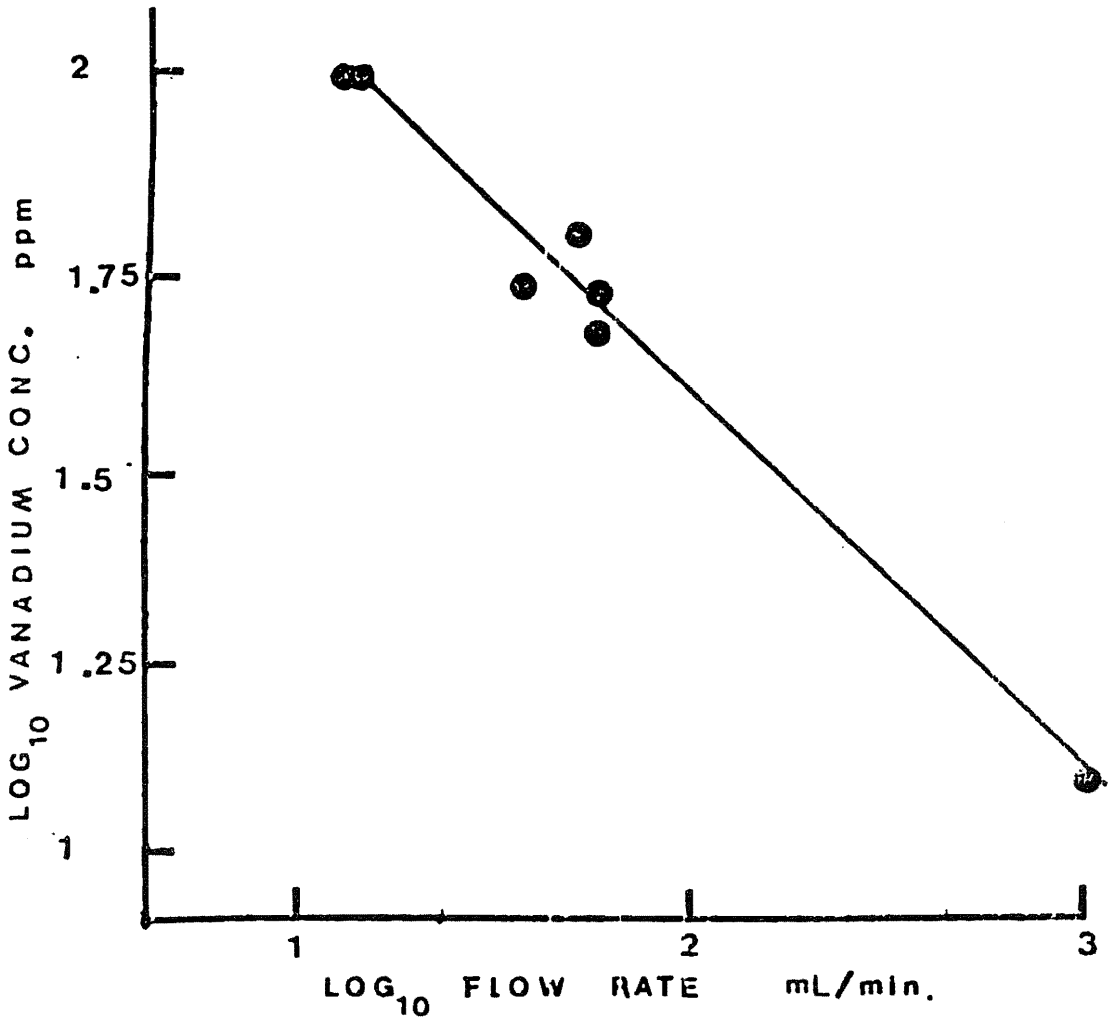

Figure 3. Concentration of total vanadium entering collecting reservoir (C in Figure 2) at different flow rates through vanadium source bottles (A in Figure 2).

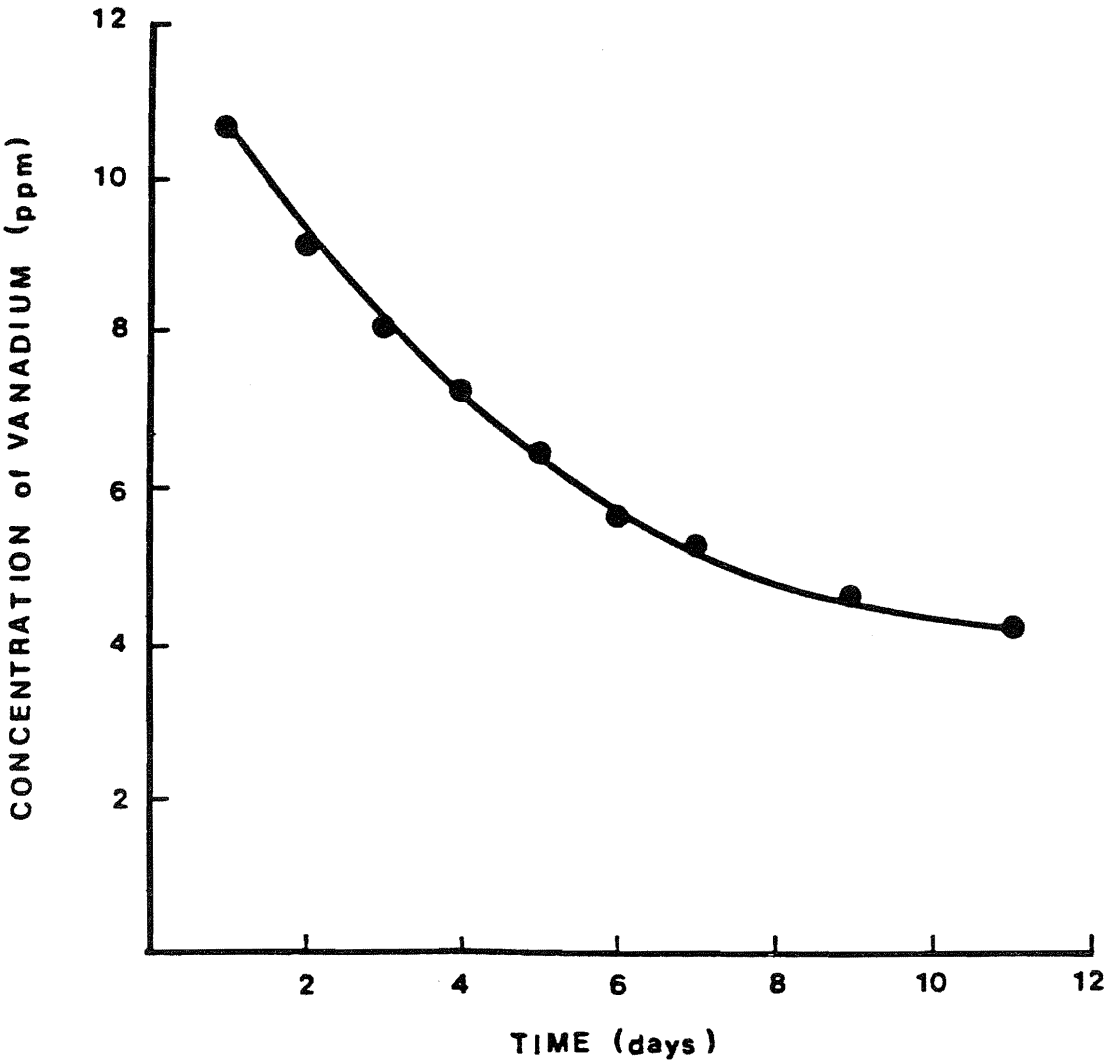

Figure 4. Decay in level of vanadium entering collecting reservoir (C in Figure 2) at specific flow rate (3 mL/min) through source (A in Figure 2) monitored over a period of several days.

adjustments for the apparent decrease in vanadium were made through an approximation: the rate of flow through the delivery system was increased by 25% daily. Comparisons of the vanadium concentrations between samples which have been collected daily from each exposure tank and assayed subsequent to the bioassay showed that such ad hoc adjustments of flow rate provided relatively constant levels of this metal throughout the course of the experiment. However, the actual mean concentration of vanadium for each tank was often significantly displaced from the desired value which best suited the testing of the multiple toxicity models. For example, in the study of the binary mixture containing vanadium and phenol (Section 4.3.4), phenol is proportionately much greater in concentration than the element. In theory, a concentration ratio that was closer to 1:1 would have been preferable. Similarly, in the binary study of nickel and vanadium (Section 4.3.3) the relative proportions of each contaminant would have prevented a discrimination between similar and independent acting models of multiple toxicity. In this particular study, however, the response data were such that neither model applied (see Sections 2.1 and 2.2).

3.3.2 Nickel and Phenol Stock Solutions

Stock solutions of nickel and phenol were held individually in Mariotte bottles and dripped at controlled rates into the dilution apparatus (Figure 2).

The nickel stock solution was prepared by dissolving 248 g of analytical grade nickelous sulphate in each 20 L of distilled water. The stock solution of phenol contained 483 g analytical grade phenol per 20 L of distilled water. The pH of both stock solutions was adjusted to 7.8 through the addition of acetate buffer.

3.3.3 Salt Water Solutions for Sublethal Studies

Salt water solutions equivalent to 20 and 50% seawater were prepared using distilled water according to the formulae

listed in Table 2. Freezing point depression tests indicated that the osmolarity of the 20 and 50% seawaters was 271 and 552 mOsm/L, respectively.

3.4 CHEMICAL ANALYSES

3.4.1 Aqueous Samples of Vanadium and Nickel

During the course of an experiment, a water sample was taken daily from each tank, acidified, and stored for later analyses by A.A. spectrophotometry. Polyethylene containers were used to hold the samples. The volume collected for each metal determination was 40 mL.

The general operating conditions of the A.A. spectrophotometer for each metal are listed in Section 9, Table 18. Any solution beyond the maximum concentration within the linear range of detection of a particular metal by A.A. spectrophotometry was diluted, as required, with distilled water.

Three determinations were made of each day's sample and the results averaged. The daily concentrations then were summed and the mean determined. The latter values, which represent the various mean concentrations to which the respective lots of fish were exposed throughout the experiment, were used in subsequent computations of dose-response relationships.

3.4.2 Aqueous Samples of Phenol

For the analysis of phenol, two samples were taken from each experimental tank: one at the beginning, and the other at the end of the 96 h bioassays. These samples were then assayed according to the Aminoantipyrene Method recommended in APHA (1976). The binary determinations for each exposure tank were averaged and the resulting values were represented as the mean ambient concentrations to which the respective lots of fish had been exposed.

Table 2. Composition of salt water.

Chemical	g/L of distilled water	
	20% seawater	50% seawater
NaCl	7.664	19.16
KCl	0.154	0.385
$MgCl.6H_2O$	1.082	2.705
$MgSO_4.7H_2O$	1.426	3.565
$CaCl_2$	0.736	0.590
$NaHCO_3$	0.440	0.100

3.4.3 Tissue Determinations of Heavy Metals

Procedures for the precise determination of vanadium and nickel in fish tissue were developed. The procedures and data for each heavy metal are reported in Section 9.

The nickel content of whole tissue of rainbow trout with no known history of exposure to trace metals was found to be 1.45 ± 0.40 µg/g. High precision was indicated by the recovery of nickel from spiked samples of tissue (100.4 ± 2.0%).

The quantity of vanadium found in whole tissue of rainbow trout with no known history of exposure to trace metals was 0.298 ± 0.14 µg/g. The percent effectiveness of the recovery procedure for vanadium in spiked samples was 99.8 ± 4%.

The quantities of nickel and vanadium, 1.5 and 0.3 µg/g, respectively, may serve as guidelines of "normalcy" against which the degree of tissue contamination that occurs in rainbow trout inhabiting receiving waters can be compared. In such a comparison, it is important to realize that the values listed above may vary with sex, age, size, and nutritional states of a fish (Love 1970). Further studies would be required to determine how close the base levels of nickel and vanadium for rainbow trout approximate those of other fish species.

The analytical procedures for vanadium (with certail modifications as stated in Section 9.1.5) were also effective in detecting vanadium at concentrations which had accumulated above control levels in the flesh of flagfish (*Jordanella floridae*) following long-term exposure to sublethal levels of this heavy metal (Sprague et al. 1978).

3.4.4 Serum Determinations for Sublethal Studies of Nickel

3.4.4.1 Blood sampling procedure. Fish, circa 400 g, selected from test tanks were anaesthetized in a solution of tricane methane sulphonate, i.e., MS222 (Sandoz), thoroughly wiped dry, and transferred to an operating board. The caudal peduncle was severed with a scalpel. Blood which flowed from the caudal artery

was collected in 3 mL centrifuge tubes. The blood was allowed to congelate for 2 h at 11°C and then it was centrifuged at medium speed in a desktop centrifuge for 15 min. The serum was removed and frozen pending further analyses. All haemolyzed samples were discarded.

3.4.4.2 Serum chloride. The concentration of chloride in serum was determined according to the method of Schales and Schales (1941) with the following modifications:

1. A 0.05 mL sample of the serum was transferred to a test tube containing 0.5 mL of distilled water; and
2. Three drops of diphenylcarbazone indicator were added and the sample titrated with mercuric nitrate, $Hg(NO_3)_2$, to a faint blue-violet endpoint using a semi-microcuvette. These data were then compared with those of a titrated NaCl standard (0.25 meg/mL) using the following equation:

$$X_c = \frac{X_s}{V_{t.s}} \times \frac{V_s}{V_{t.b}} \times \frac{V_a}{V_{b.}} \times 1000 \qquad (7)$$

where, X_c = chloride concentration in serum (mM)

X_s = concentration of NaCl standard

$V_{t.s}$ = titration volume for standard

V_s = volume of titrant ($HgNO_3$)

$V_{t.b}$ = titration volume for serum

V_a = volume of standard aliquot

V_b = volume of serum

3.4.4.3 Serum osmolarity. An aliquot of 0.1 mL of serum was placed in a "mini-sample" cuvette and the osmolarity determined using a freezing point depression osmometer (Advanced Instruments, Model 67-312AS).

3.4.4.4 Serum protein. Serum protein was assayed using a Lowry protein assay according to a modification of Hesser (1960). A 5 mL serum sample, rather than a 0.5 mL sample, was used. The levels of protein, with ranges from 100 to 600 µg/mL, then can be analyzed through the use of this method.

3.4.5 General Bioassay Procedures.

3.4.5.1 Lethal studies. Twenty-four hours prior to each experiment, 80 fish were randomly selected from holding tank cultures, wet weighed (Table 3), and divided into 10 lots of eight fish. Eight, rather than the more standard number of 10 fish were used because of the limited size of the test tanks. Weighing operations were conducted under red light to reduce handling stress. Each lot was held separately under control conditions until the following day. At that time, the lots of fish were transferred to 10 bioassay tanks which had been pre-set to experimental conditions. Fish were not fed from the time of weighing through to the end of 96 h of exposure. From the fifth day on, in incipient lethal studies, fish were offered a daily maintenance diet equivalent to 2% of their body weight.

Times at which observations were made during the course of experiments were frequent and varied depending on the contaminant. The criteria for death were a lack of opercular movement and unresponsiveness to tactile stimuli. For certain experiments, control fish were removed at the end of the experiment, dried at 65°C for five days, and then weighed. These dry weight values are compared to wet weights in Table 3.

The guidelines recommended for bioassays by APHA (1976) and Sprague (1973) were followed except for the temperature which was 12 ± 1°C rather than the specified 15°C. The pH of control water and test solutions ranged from 7.8 to 8.0.

Table 3. Fresh and dry weights of rainbow trout used in experiments (n = 8).

Study	Average Wet Weight	Average Dry Weight
Vanadium	31.2 ± 5.5[a]	5.8 ± 1.2
Nickel	29.4 ± 5.2	5.6 ± 1.1
Phenol	28.0 ± 7.1	
Nickel and Vanadium	23.2 ± 5.6	4.2 ± 1.4
Vanadium and phenol	28.2 ± 6.1	
Tertiary mixture	22.8 ± 4.8	

[a]Standard deviation

Time-response curves were developed and have been submitted in an interim report (Spear and Anderson 1977). From these graphs, the LT_{50}'s were determined over a range of concentrations for each toxicant. These LT_{50}'s were then plotted against their respective concentrations to derive toxicity curves for the respective toxicants.

For 96 h exposure periods, dose-response derivations were computed in accordance with a combined "working" and "weighted" probit procedure (Finney 1971). The probit program was run on Concordia's computer and provided for each set of data a probit regression function, a median lethal concentration (LC_{50}), the latter's standard deviation, a correlation coefficient, and a Chi square test.

3.4.5.2 Sublethal studies. A series of nickel solutions, representing concentrations of 0.8, 2.8, and 4.6 mg/L, were selected for the sublethal tests. These levels are related to the 96 h LC_{50}, i.e., 28 mg/L (Section 4.1.3) by "application" factors of 0.028, 0.098, and 0.16, respectively. EPA (1976) suggest that nickel concentrations at or less than 0.01 of the 96 h LC_{50} are "safe" for freshwater life.

Sixteen fish were tested at each concentration level. The exposure tanks were polyethylene and had a holding capacity of 285 L. The solutions of nickel were continuously delivered to the tanks by the dilution apparatus described in Section 3.2. The flow rate insured that 95% of the solutions were exchanged within 6 h and thus met the recommended guidelines (Sprague 1969).

Four fish from each lot, at exposure intervals of 26, 45, 74, and 104 h, were removed for blood analyses. Unaccountably, two of four fish in a test tank which contained 4.6 mg/L died following 74 h of exposure. In contrast, fish which were subjected to a similar level of nickel, but in a static bioassay, showed no signs of lethal stress (see below).

The salt-stress experiments were run under static conditions. Salt solutions in tanks were replaced at 64 h; nickel solutions were replaced daily. Temperature was controlled by circulating water at 12°C through stainless steel coils immersed in the tanks. Air stones held oxygen levels above 85% saturation.

Two lots, consisting of 18 fish each, were transferred abruptly from control conditions to saline bath solutions equivalent to 20 and 50% seawater (Table 2). Four fish were removed from each of the two salt baths for blood analyses following exposures of 5, 26, 45 and 104 h; only two fish were sampled at 72 h. An additional lot of 14 fish remained under control conditions in freshwater.

A similar static bioassay was subsequently performed in which fish were exposed to 5.14 ± 0.30 mg/L of nickel for 48 h prior to their being transferred to saline solutions equivalent to 20 and 50% seawater. Sampling numbers and times were identical to the former experiment on salt stress.

3.4.6 Procedure for Testing Multiple Toxicity of Certain Mixtures of Vanadium, Nickel, and Phenol

3.4.6.1 Derivation of quantal response curves. Preparation and operation of the toxicity apparatus which provided a range of lethal concentrations within a series of exposure tanks were carried out in accordance with Section 3.2. The procedures for handling fish and composing test lots are described in Section 3.1.

Fish mortality, in percentage deaths per lot, was recorded following a 96 h exposure period in bioassays of discrete toxicants and of their mixtures and was adopted as the measured, quantal-response criterion. Linear regression, which represents probit mortality as a function of the $\log_{10}$ concentration of certain mixtures and each individual toxicant, was derived through a computer program that combined, in sequence, the working and weighted probit analyses of Finney (1971).

Probit-derived, linear regressions can be represented by the general equation,

$$Y = y^1 + b \log_{10} X \qquad (8)$$

where Y = probit mortality at concentration X

y^1 = ordinate intercept

b = regression coefficient (i.e., slope)

X = mean (daily) concentration of toxicant or toxic mixture in mg/L

This straight line representation of the relationship between quantal response and toxicant concentration is useful for several purposes, some of which are considered below.

Firstly, the regression coefficients of lines can be compared. Parallel slopes indicate that the distribution of tolerances within a test population is the same for the respective toxicants. According to the model, this analogy in slopes suggests that the individual toxicants may be acting at identical target sites within an organism.

Secondly, it is possible to readily compute a concentration threshold to lethal response. Arbitrarily, this threshold was set at that concentration below which 99.99% of the population did not respond. This threshold variable is to be considered as a mathematically derived value which serves certain quantitative and comparative purposes. Where two or more regression lines are parallel, this threshold value may be used to test whether the lines are significantly different.

Finally, specific measures of toxic potency can be calculated from the quantal response function (8). For example, the LC_{50} is accepted as a standard measure of lethal potency between toxicants. In this approach to multiple toxicity, this comparison was extended to include the full range of quantal responses between 0.01 and 99.99% mortality. Then, to facilitate an appreciation of the relative contribution of each toxicant as

a constituent of a lethal mixture, all values were converted to "vanadium-equivalent" units. Vanadium was arbitrarily selected as the reference toxicant. For example, the LC_{50} of nickel, 28.6 mg/L, becomes 11.52 mg/L vanadium-equivalents (Section 4.3.2).

3.4.6.2 Derivation of vanadium-equivalent potency units. The methods of converting nickel and phenol concentrations to equivalent vanadium units are as follows.

When dose-response lines are parallel, as in this study between vanadium and nickel, then the potency factor can be derived simply by comparing the LC_{50}'s;

$$\frac{LC_{50}A}{LC_{50}B} = P \qquad (9)$$

where $LC_{50}A$ = median lethal concentration of the reference toxicant A

$LC_{50}B$ = median lethal concentration of an elect toxicant B

P = potency factor

A potency factor of 0.403 transposes dose-response data of nickel to vanadium equivalents. Such a potency factor is a constant that can be applied throughout and beyond (below) the dose-response range.

When dose-response lines are not parallel, as in the study between vanadium and phenol, then the potency factor is derived by using values from equation (8) to solve for I in the following manner:

$$\frac{I - YA^1}{b_A} = \frac{I - YB^1}{b_B} \qquad (10)$$

where YA^1 = ordinate intercept of the linear regression for the standard toxicant (e.g., vanadium)

YB^1 = ordinate intercept of the linear A regression for a toxicant (e.g., phenol).

b_A = regression coefficient of standard toxicant (e.g., vanadium)

b_B = regression coefficient of elect toxicant (e.g., phenol)

I = the ordinate value at which the linear functions of standard and elect toxicant intercept

The value I is then introduced into the following formula:

$$\log_{10} X = \log_{10} X_B - (I-Y) \frac{1}{b_A} - \frac{1}{b_B} \quad (11)$$

where X = concentration of an elect toxicant B (e.g., phenol) in equivalent potency units of standard (e.g., vanadium)

X_B = concentration of an elect toxicant B (e.g., phenol) which involves a particular magnitude of response (Y)

Y = the magnitude of response (in probits) evoked by X_B

3.4.6.3 Test for strict addition. When the linear regressions of dose-response data for two or more discrete toxicants are parallel, it is assumed that these poisonous agents are similarly acting and therefore, as constituents of mixtures, such toxicants obey the principles of strict addition (Section 2.2). This hypothesis is tested by comparing the linear regression fitted to the observed response co-ordinates of the mixture to the predicted linear regression as represented by the following equation:

$$Y_m = Y_A^1 + b \log 10 (X_A + X_B + \ldots X_i) \quad (12)$$

where Y_m = predicted probit mortality for the mixture

Y_A^1 = ordinate intercept of the standard toxicant (i.e., vanadium)

b = common slope to dose-response curves of each constituent
(For simplification, the value assigned was the regression coefficient of the standard toxicant's dose-response curve.)

X_A, X_B...X_i = concentration of each toxicant in toxicant A - equivalent units (Section 3.4.6.2). In these experiments, vanadium was arbitrarily designated as toxicant A.

The basic premise upon which a series of mixtures is prepared is that the sum of the respective concentrations (in vanadium-equivalent units) of each constituent in a mixture should fall within the lethal response range of vanadium as a discrete solution. Furthermore, the series of mixtures so composed should represent a distribution of vanadium-equivalent concentrations that extends over the lethal response range of vanadium as a discrete solution. The proportion of each constituent within a mixture is a matter of choice. Multiple toxicity studies have traditionally tested mixtures with equal or approximately equal proportions (e.g., 1:1) between constituents. However, constant proportions between constituents limit the evaluation of interactions which might occur in a form of either antagonism or synergism (Sections 2.3 and 2.4). The actual amounts of X_A, X_B ...X_i are determined by a chemical assay of daily samples taken from solutions in test tanks during the 96 h bioassay of mixtures.

3.4.6.4 Test for response addition. If regression coefficients of dose-response curves for two or more toxicants are significantly different ($P > 0.05$) or, if the known differences in toxic action between toxicants warrant the surmise that the similarity in slopes of their dose-response curves is coincidental, then such toxicants are assumed to be independently acting, i.e., their toxic actions ensue at different target sites within an organism. Independently acting toxicants as constituents of mixtures may contribute to a

common effect in accordance with the empirical model of response addition (Section 2.1). The percent mortality caused by a mixture whose constituents are response additive is predicted by the following formula which assumes that the tolerance distributions are random and similar for the respective constituents:

$$P_m = 100 - \frac{(100-P_A)\ (100 - P_B)}{100} \qquad (13)$$

where P_m = percent mortality within fish lots exposed to a mixture

P_A and P_B = the proportions (as percent mortality) of each test lot that respond to pure solutions of toxicity and A and B respectively at concentrations equivalent to those prevailing for each in the mixture

It is important to appreciate the fact that, in response addition, only poisonous constituents whose concentrations are above threshold contribute to the common response. Upon conclusion of a 96 h bioassay of a series of response additive mixtures, the predicted response in each tank, as determined by formula (13) and in accordance with assayed concentrations, is compared to the respective observed response using a Chi Square test for significant differences.

Regression lines can be fitted to dose-response data for response additive mixtures according to formula (13). However, before a curve is fitted, the "effective" concentration of a mixture is computed as a function of the amount of each constituent that exists above the thresholds of their respective dose-response curves (equation 14). The following formula defines the "effective" concentration of the mixture in vanadium equivalent units.

$$\log Cm = \log M - \frac{(\log \frac{M}{A})\ (\log \frac{M}{B})}{\log \frac{M}{T}} \qquad (14)$$

if A or B $<$ T, then A or B = 0

if A or B $>$ M, then A or B = M

where Cm = the "effective" concentration in vanadium-equivalents, of a response additive mixture

M = concentration of vanadium which in pure solution evokes 99.9% mortality

A = concentration, if any, of vanadium in mixture

B = concentration of nickel or phenol in equivalent vanadium units

T = threshold concentration of vanadium in pure solution

In effect, this formula converts the combined concentration of toxic constituents which are response additive to the amount of vanadium in pure solution that elicits an equivalent response.

3.4.6.5 Synergisms and antagonisms. When the magnitude of response to mixtures is other than predicted by either strict or response additive models, it is assumed that the individual toxicants have interacted. Interactions which result in a response less than that predicted are termed antagonisms; interactions which result in an enhanced response to that predicted are termed synergisms. In this study, no attempt was made to explain the mode of interactions which lead to non-additive effects. Rather, the extent of an antagonism or synergism was arbitrarily compared to the response magnitude predicted for strict and/or response addition. In this approach, antagonisms are qualified by the term infra-additive and synergisms by the term supra-additive. Furthermore, because the degree of interaction is often related to the relative proportions of each constituent within a mixture, response patterns for these non-additive events are examined as a function of the ratio between the concentration of an elect constituent and the total concentration of the mixture.

4. RESULTS

4.1 LETHAL TOXICITY

The time to mortality data for juvenile rainbow trout exposed to pure solutions of vanadium, phenol, and nickel are presented in Tables 4, 5, and 6, respectively. These data are plotted in Figure 5.

4.1.1 Lethal Toxicity of Vanadium

The observation period for vanadium-exposed fish was 2 wk during which there was no indication of an incipient lethal threshold. The lowest concentration of vanadium at which at least 50% of the fish died within the 14 d period was 2.8 mg/L. In comparison, the 7 d LC_{50} was estimated as 4.8 mg/L and the 4 d LC_{50} was 11.5 mg/L. Survivors of the 14 d experiments were removed from their respective test chambers, held collectively in freshwater, and observed occasionally during subsequent weeks. Although no longer exposed to vanadium in their ambient environment, many of these experimental fish died.

The most evident and consistently occurring signs associated with vanadium poisoning in these lethality tests were loss of appetite, a high frequency of coughing, and a disruption of balancing or stabilizing functions which are required for normal swimming. The latter dysfunction was often accompanied by a vertical, "heads up", posture. Vanadium-stressed fish also showed an increased susceptibility to bacterial infection, e.g., tail and fin rot, and they frequently developed opaqueness in the corneal layer of their eyes.

4.1.2 Histopathology of Vanadium Poisoning

Histological examination was carried out on gill, intestine, liver, spleen, and kidney tissues from control and vanadium-treated fish. The only detected lesions were extensive

Table 4. Time-mortality data for rainbow trout exposed to vanadium.

Vanadium concentration (ppm)	Time to mortality (h) for each increment of cumulative percent mortality within a log of eight fish								Estimated time to 50% mortality (h) at various concentrations of vanadium
	12.5	25	37.5	50	62.5	75	87.5	100	
0.0	--	--	--	--	--	--	--	--	--
2.8	165	195	235	288	312	340	--	--	270
3.3	195	--	--	250	262	272	340	--	263
3.5	202	212	--	220	224	288	--	--	221
3.7	177	192	212	235	242	--	--	262	224
4.5	165	177	192	199	250	288	340	--	224
4.8	--	153	165	--	177	192	212	262	173
5.4	128	137	165	195	--	--	212	235	173
12.6	--	34	46	--	80	96	--	--	65
15.2	--	34	--	--	46	80	96	--	53
15.2	--	26	--	46	--	--	80	96	48
24.2	11	26	34	46	--	--	52	80	38
25.1	11	--	--	--	26	--	--	34	22

Table 5. Time-mortality data for rainbow trout exposed to phenol.

Phenol concentration (ppm)	Time to mortality (h) for each increment of cumulative percent mortality within a lot of eight fish								Estimated time to 50% mortality (h) at various concentrations of phenol
	12.5	25	37.5	50	62.5	75	87.5	100	
0.0	--	--	--	--	--	--	--	--	--
8.3	19	--	--	--	--	--	--	--	--
9.9	--	19	--	--	--	--	--	--	--
10.2	--	--	19	22	24	--	--	--	21.5
11.4	--	9	--	--	--	--	--	19	15
13.4	--	--	7	9	--	--	--	19	10.4
14.6	4	--	--	7	--	9	--	19	7.8

Table 6. Time-mortality data for rainbow trout exposed to nickel.

Nickel concentration (ppm)	Time to mortality (h) for each increment of cumulative percent mortality within a lot of eight fish								Estimated time to 50% mortality (h) at various concentrations of vanadium
	12.5	25	37.5	50	62.5	75	87.5	100	
0.0	--	--	--	--	--	--	--	--	--
40.9	43	45	--	49	57	66	70	80	53
36.9	49	57	66	70	80	--	92	141	72
34.0	57	66	--	--	92	104	112	140	82
31.8	43	49	51	68	70	73	92	130	62
30.5	44	--	66	104	--	112	130	141	84
27.4	46	70	73	--	92	130	167	192	87
26.4	80	104	112	118	121	--	141	192	115
23.2	51	--	118	--	130	--	141	192	125
21.8	66	92	98	112	141	190	215	--	120

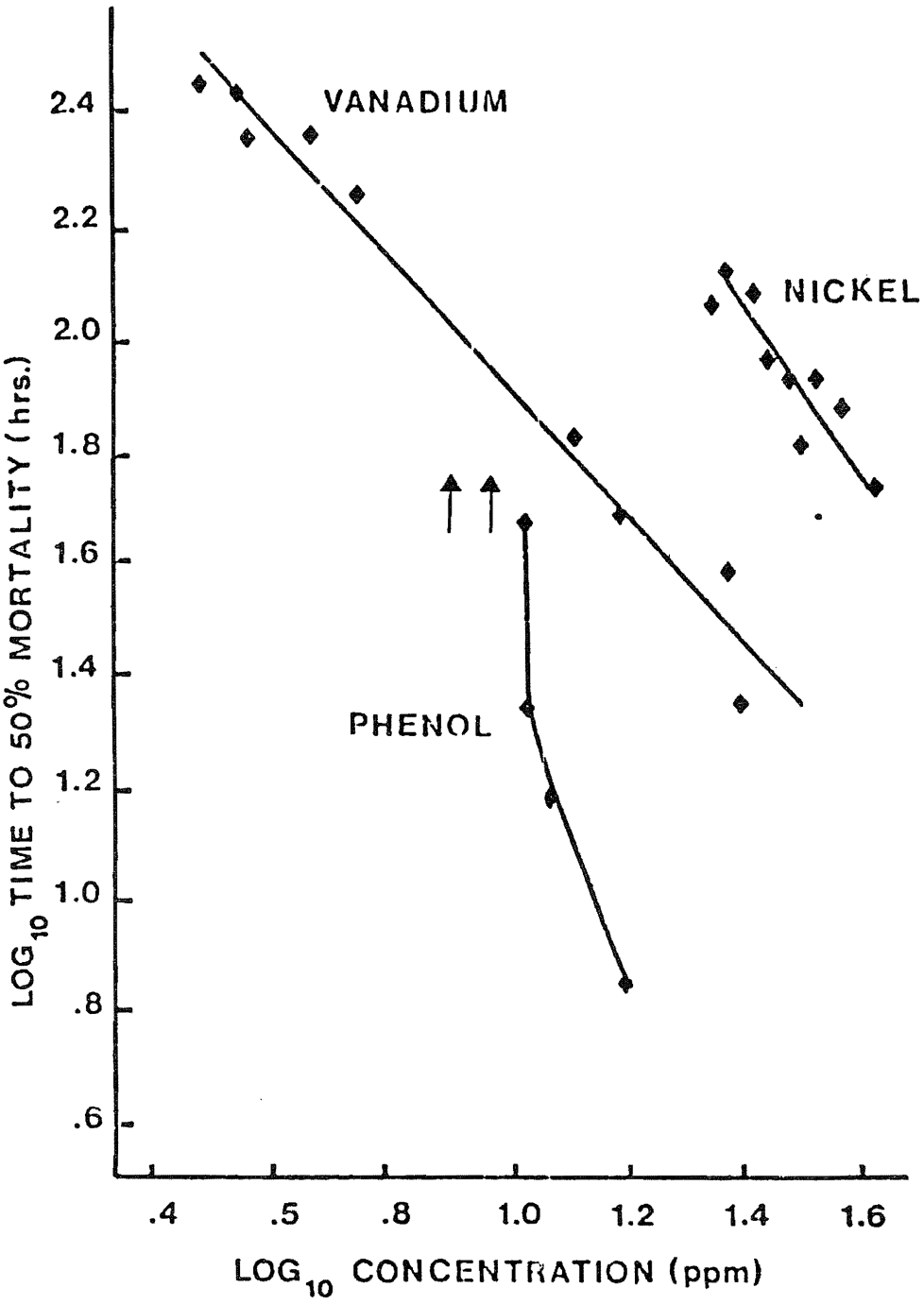

Figure 5. Effect of toxicant concentration on median survival time for rainbow trout exposed to either phenol, vanadium, or nickel. Arrows (↑) indicate concentrations at which less than 50% response was observed over the duration of experiment.

damage to the secondary lamellae of gills. The secondary lamellae from gill filaments of control fish are shown in Figures 6 and 7. Gill sections from vanadium-exposed fish showed a lifting of the epithelium from the basement membrane of the pillar cell system accompanied by an increase in the width of the subepithelial space, damage being most extensive in gill filaments at the dorsal and ventral edges of the gill (Figures 8 and 9) with least damage to central filaments (Figures 10 and 11). The average width of the subepithelial space of secondary lamellae (see Figure 8) from the least affected filaments (90 determinations, 9 lamellae each from 10 filaments) increased from 0.019 ± 0.002 mm to 0.050 ± 0.008 mm. The epithelia of secondary lamellae in the filaments at the dorsal and ventral edges of the gill had been so extensively elevated that only remnants of epithelium remained above marginal endothelial cells (Figures 10 and 11).

4.1.3 Lethal Toxicity of Phenol

The toxicity curve for phenol-exposed trout contrasts with those of vanadium and nickel because it displays an incipient lethal level of 10.2 mg/L after only 21.5 h exposure (Figure 4).

Fish exposed to lethal amounts of phenol demonstrated erratic locomotory movements, periodic spasms in the trunk musculature, and eventual loss of swimming ability. The seemingly paralyzed specimens settled to the bottom of the aquaria where they would lie motionless except for opercular movement.

4.1.4 Lethal Toxicity of Nickel

Lethal response data were collected over a period of 10 d for nickel-exposed fish (Table 6). The concentration range selected for this experiment did not allow for the empirical determination of an incipient lethal level. If the toxicity curve for nickel shown in Figure 5 is extrapolated to 10 d, then the

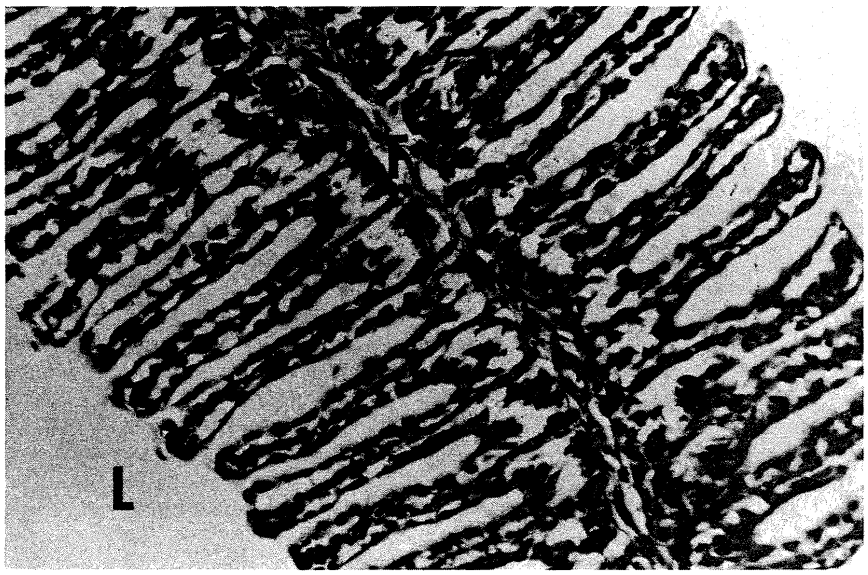

Figure 6. Photomicrograph of a transverse section of a control gill showing one primary filament (F) with secondary lamellae (L) (X300).

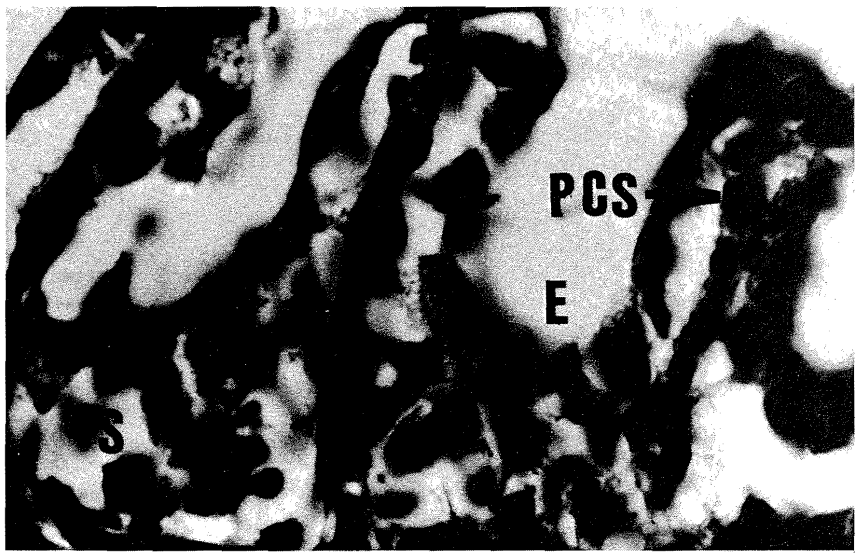

Figure 7. Higher magnification of above section showing three secondary lamellae (L) consisting of a pillar cell system (P.C.S.), epithelial cells (E) attached to the basement membrane (B.M.), and a subepithelial space (S) (X1200).

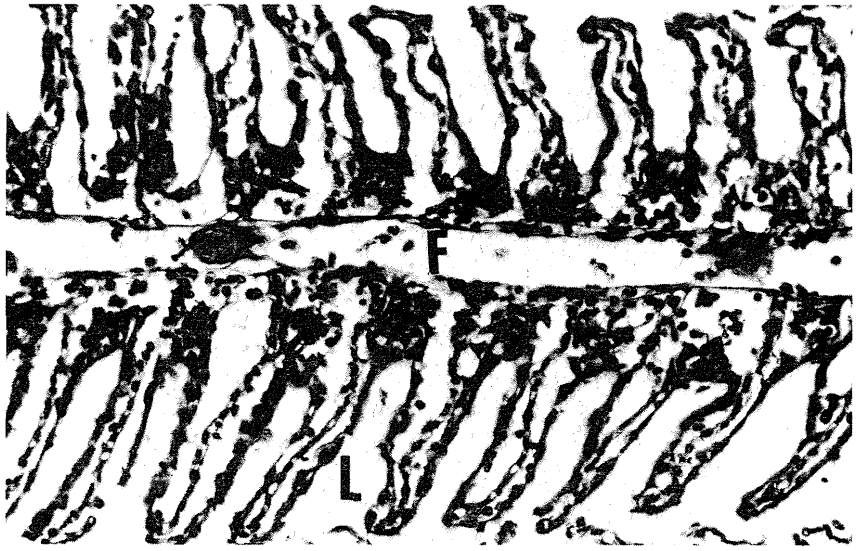

Figure 8. Photomicrograph of a transverse section of a gill, from rainbow trout exposed for 96 h to vanadium, showing a central primary filament (F) with secondary lamellae (L) (X300).

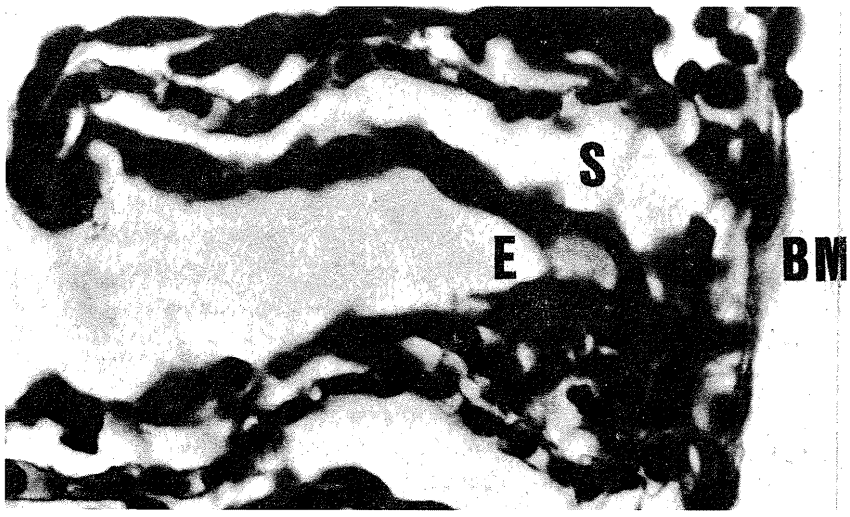

Figure 9. Higher magnification of above section showing a lifting of the epithelium (E) from the basement membrane (B.M.), resulting in an enlargement of the subepithelial space (S) (as seen in Figure 8) (X1200).

Figure 10. Photomicrograph of a transverse section of a gill, from rainbow trout exposed for 96 h to vanadium, showing a distal primary filament (F) with secondary lamellae (L) (X300).

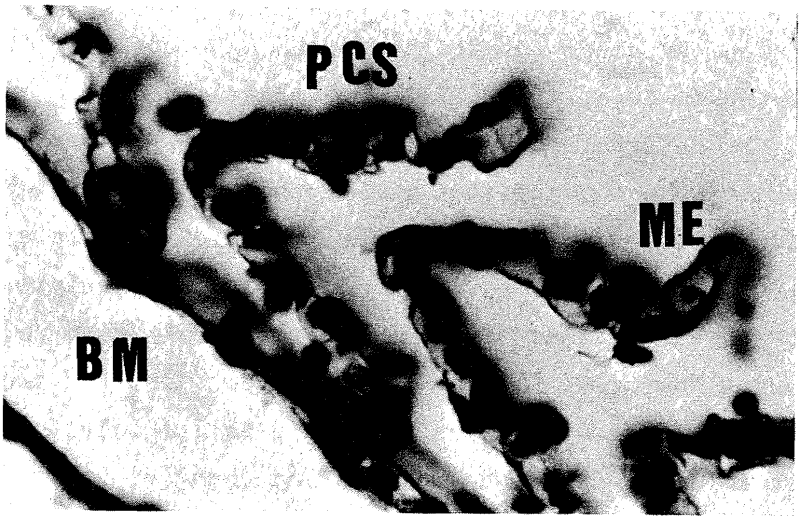

Figure 11. Higher magnification of above section showing the remaining pillar cell system (P.C.S.). The epithelial cells have been detached from the basement membrane (B.M.) so that only remnants remain (as seen in Figure 10) attached to marginal endothelial cells (M.E.) (X1200).

estimated LC_{50} is approximately 15 mg/L. This computation is only acceptable on the assumption that an incipient lethal level does not occur between 15 and 21.9 mg/L. The latter concentration caused deaths in 50% of the test lot in five days and in 87.5% of the organisms by the ninth day.

The 96 h LC_{50} computed from the nickel toxicity curve is approximately 28 ± 1.07 mg/L. Yet in the studies of the effects of nickel on osmoregulation (Section 4.2), two of four fish, in a lot which had been exposed to 4.67 mg/L of nickel for 4 d, died; the remaining individuals were moribund. No explanation for this dramatic difference in 96 h LC_{50} values can be given except that fish in the osmoregulatory experiments were more than 10 times the size of those used in the formerly described lethality tests.

4.2 THE EFFECTS OF NICKEL ON OSMOREGULATION

Values for serum chloride (117 ± meg/L of blood) and osmolarity (233 ± 20 mOsm/L of blood) in control fish were similar to those found by other investigators who have worked with salmonids (McKim et al. 1970; Potts and Parry 1963).

The external morphology and general behaviour of the rainbow trout were not apparently altered when fish, which had been acclimated to freshwater, were abruptly challenged by a saline stress equivalent to 50% seawater. However, serum chloride and osmolarity levels increased rapidly to about 35% above control values during the first 75 h in the seawater (Tables 7 and 8 and Figures 12 and 13). Direct transfer to a saline solution equivalent to 20% seawater caused similar changes, but all responses were less in magnitude.

Pre-exposure of rainbow trout to a 5 ppm nickel solution prepared in freshwater did not appear to affect the serum chloride or osmotic responses at either level of salt stress (Tables 7 and 8 and Figures 12 and 13). In both the 50 and 20% equivalent seawater, the patterns of chloride and osmotic adjustment are not significantly different from those of control fish except for two points.

Table 7. Effect of 2-day pre-exposure of 5 ppm nickel on serum chloride level in rainbow trout upon exposure to 20 and 50% seawater.

Time of exposure (h)	Chloride level (meq/L)			
	20% seawater		50% seawater	
	Control	5.14 ppm nickel	Control	5.14 ppm nickel
0	117 ±4[a](12)[b]	----------	117 ± 4(12)	----------
5	122 ±3(4)	122 ± 3(3)	132 ± 5(3)	136 ± 13(3)
26	126 ±2(4)	[c]129 ± 2(4)	148 ±11(3)	145 ± 9(4)
45	128 ±3(3)	128 ± 9(4)	159 ±15(4)	143 ± 14(3)
74	130 ±1(2)	[c]137 ± 1(2)	144 ± 6(2)	138 ± 16(2)
104	133 ±3(4)	[c]124 ± 6(4)	147 ±14(4)	137 ± 21(4)

[a]standard deviation
[b]number of observations
[c]significantly different from control at $p<0.05$

Table 8. Effect of 2-day pre-exposure of 5 ppm nickel on serum osmolarity in rainbow trout upon exposure to 20 and 50% seawater.

Time of exposure (h)	Osmolarity (mOsm/L)			
	20% seawater		50% seawater	
	Control	5.14 ppm nickel	Control	5.14 ppm nickel
0	233 ± 20[a](13)[b]	----------	233 ± 20(13)	----------
5	246 ± 8(4)	226 ± 48(3)	260 ± 11(3)	[c]309 ± 9(3)
26	255 ± 31(4)	227 ± 30(4)	279 ± 53(3)	302 ± 31(4)
45	248 ± 19(3)	258 ± 13(4)	313 ± 30(4)	298 ± 35(3)
74	255 ± 0(2)	231 ± 33(2)	295 ± 6(2)	281 ± 41(2)
104	255 ± 2(4)	249 ± 25(4)	288 ± 21(4)	284 ± 46(4)

[a] standard deviation
[b] number of observations
[c] significantly different from control sample at $p<0.05$

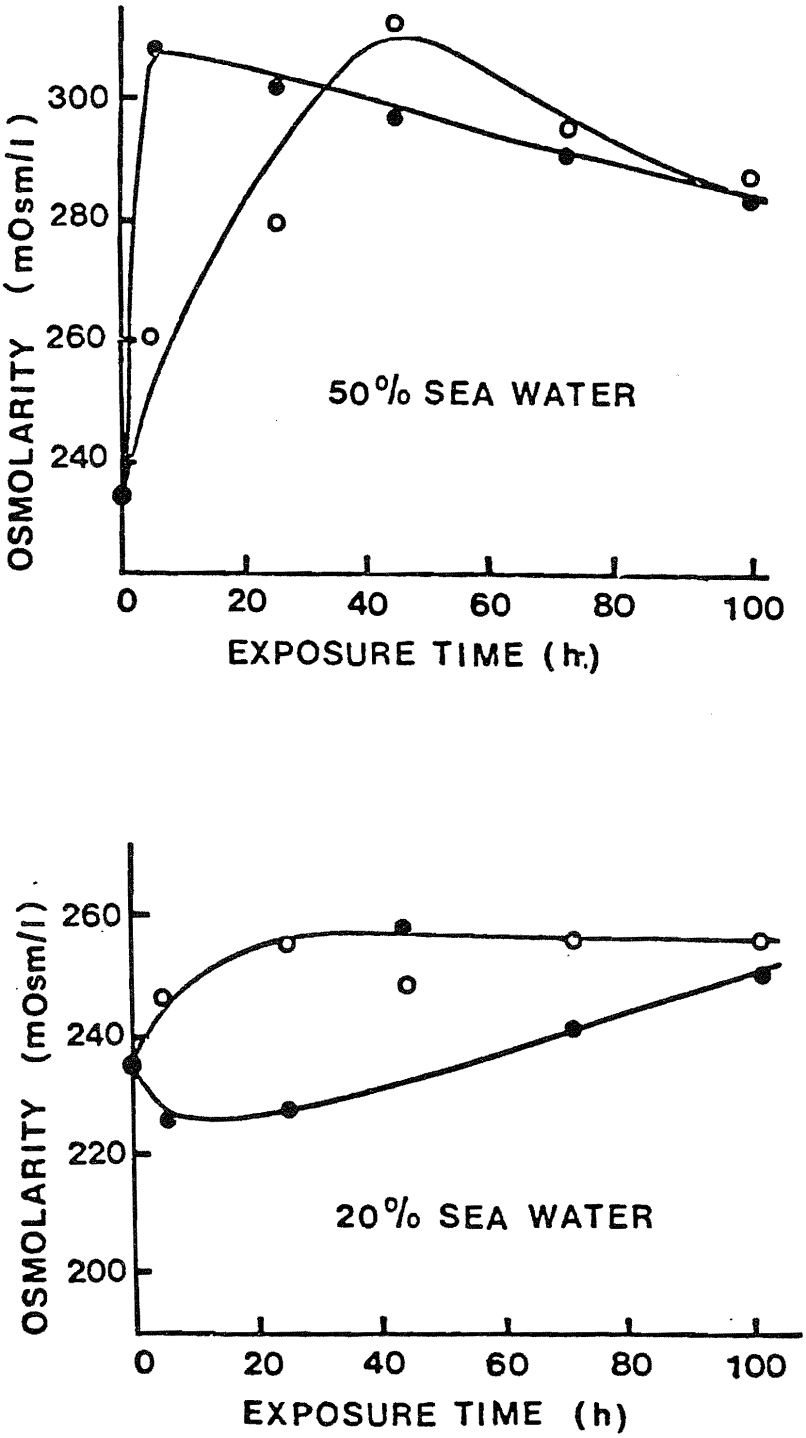

Figure 12. Changes in serum osmolarity in rainbow trout upon their transfer to 20 and 50% seawater, with (●) and without (○) prior nickel exposure.

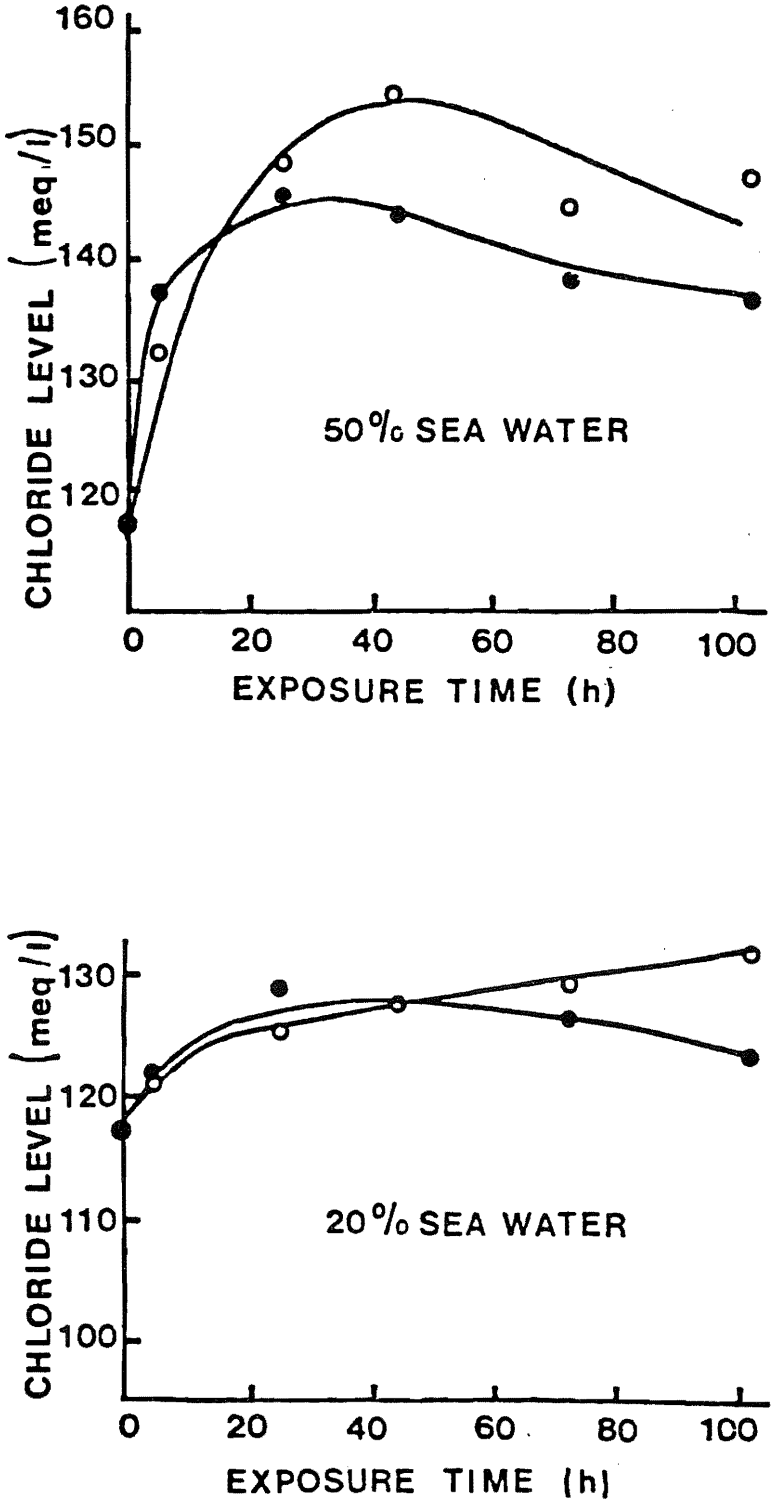

Figure 13. Changes in serum chloride levels in rainbow trout upon their transfer to 20 and 50% seawater, with (●) and without (○) prior nickel exposure.

There were significant increases ($P \leq 0.05$) in serum protein content in fish exposed continuously to 0.8, 2.8, and 5 ppm nickel in freshwater, with the increases occurring earlier at higher nickel concentrations (Table 9 and Figure 14). The increases in serum protein seem to be dose-related. In addition, there were significant increases in serum osmolarity in those fish exposed to 2.8 and 5 ppm nickel ($P \leq 0.05$). The eventual displacements in osmolarity were about equal at both concentrations (Table 10 and Figure 15). The serum chloride levels in fish exposed to 2.8 ppm nickel decreased slightly. In contrast, the chloride concentration increased significantly at the strongest nickel solution of 5 mg/L ($P \leq 0.05$) (Table 11 and Figure 16).

4.3 MULTIPLE TOXICITY

4.3.1 Dose-Response Curves

Tables 12, 13, and 14 document the dose-response data of cumulative percent mortality at 96 h of exposure and $\log_{10}$ concentrations of pure solutions of vanadium, nickel, and phenol. The linear regression functions, which were derived for each toxicant according to the weighted-probit procedure of Finney (1971), are given at the bottom of the tables. The correlation coefficients (r) suggest that the probability of points not fitting a linear distribution is no greater than 4% for vanadium, 20% for nickel, and 12% for phenol. The three functions are plotted in relation to their respective dose-response co-ordinates in Figure 17.

4.3.2 Relative Lethal Potency

The probit regression functions (Tables 12, 13, and 14) are useful in computing the standard criteria of lethal potency, i.e., 96 h LC_{50} (Sprague 1969). The 96 h LC_{50} is 11.52 ± 0.73 mg/L for vanadium, 28.60 ± 1.07 mg/L for nickel, and 10.03 ± 0.24 mg/L for phenol. There is a suggestion, due to the slopes of the curves, that vanadium is the most potent of the three agents when less than 20% of the population is considered.

Table 9. Percent changes in serum protein content in rainbow trout exposed to nickel.

Time of exposure (h)	Percent of control serum protein content at various nickel concentrations (ppm)		
	0.8	2.8	4.64
0	100 ±10.8[a](24)[b]	100 ±14.4(21)	100 ±25.6(13)
24-48	100 ±10.8(6)	[c]112 ± 7.5(5)	122 ±25.6(13)
45-48	102 ±10.5(6)	110 ± 7.3(6)	123 ±15(3)
74	----------	----------	[c]129 ± 8.7(5)
94-104	[c]114 ±10.2(6)	[c]121 ±14 (6)	130 (1)
192	[c]120 ±10.5(6)	[c]121 ± 6.6(6)	----------

[a]standard deviation
[b]number of observations
[c]significantly different from 0 h sample at $p<0.5$

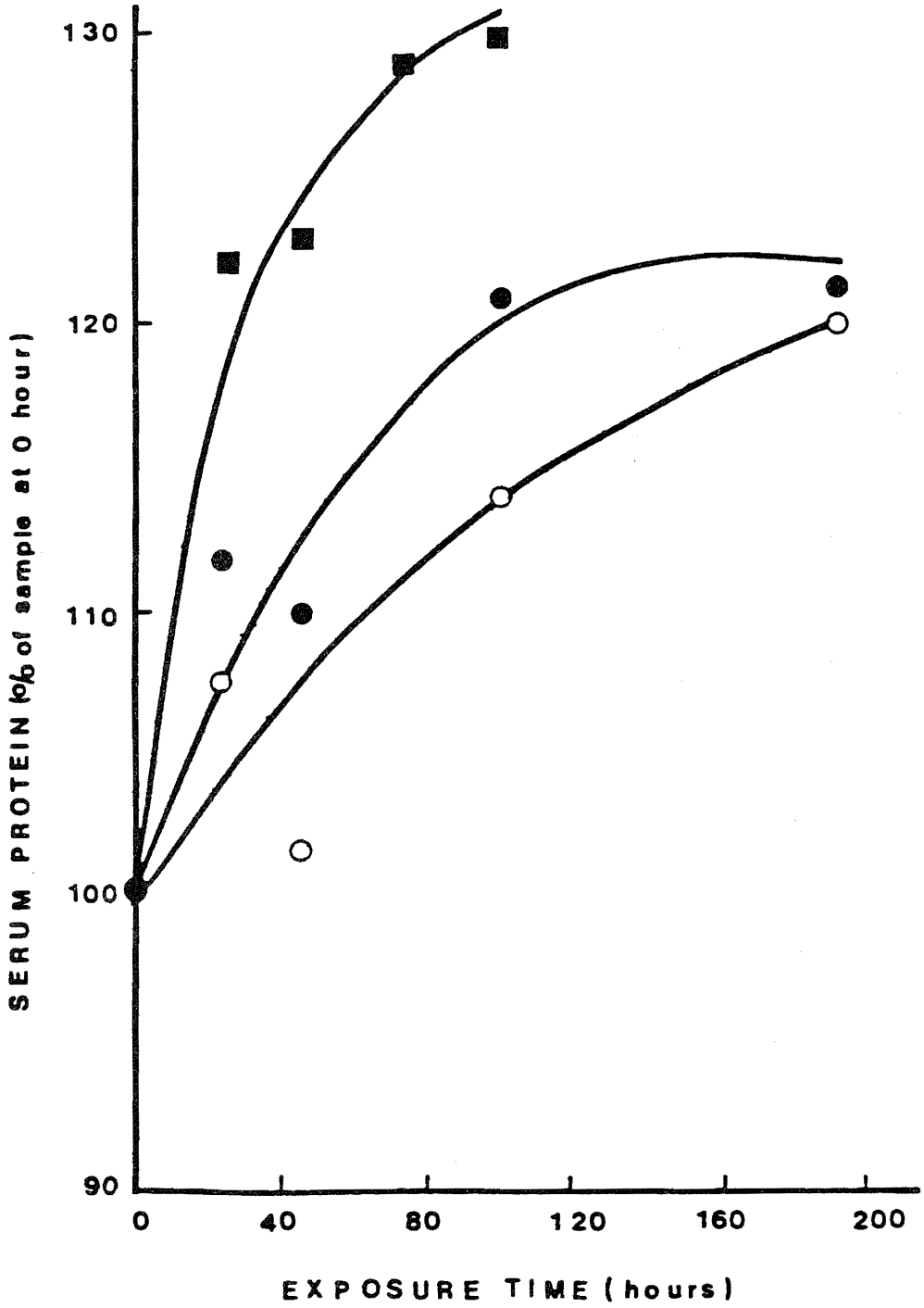

Figure 14. Changes in serum protein level (as % of value to time 0) in rainbow trout upon their transfer to 0.8 (○), 2.8 (●), and 4.6 (■) ppm nickel.

Table 10. Percent changes in serum osmolarity in rainbow trout exposed to nickel.

Time of exposure (h)	Percent in serum osmolarity at various nickel concentrations (ppm) 0.8	2.8	4.64
0	100 ± 1.6[a](24)[b]	100 ± 12.4(21)	100 ± 8.6(13)
24-26	99 ± 0.7(6)	[c]113 ± 6.9(5)	106 ± 8.6(4)
45-48	100 ± 1.1(6)	[c]110 ± 4.4(6)	109 ± 7.3(3)
74	N.D.	N.D.	[c]110 ± 3.4(3)
94-104	101 ± 1.0(6)	[c]114 ± 7.0(6)	114 ± (1)
192	100 ± 1.1(6)	108 ± 9.2(6)	N.D.

[a]standard deviation
[b]number of observations
[c]significantly different from 0 h sample at $p<0.5$
N.D. no determinations

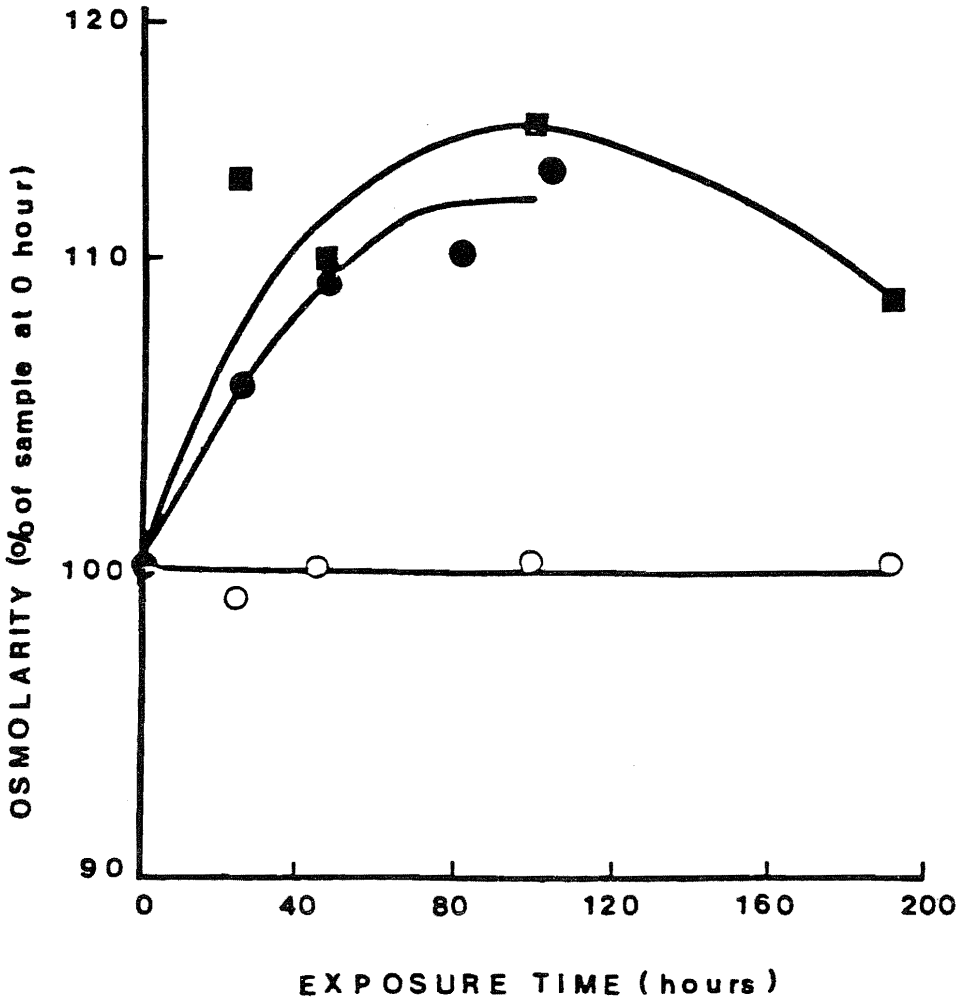

Figure 15. Changes in serum osmolarity (as % of value at time 0) in rainbow trout upon transfer to 0.8 (○), 2.8 (●), and 4.6 (■) ppm nickel.

Table 11. Percent changes in serum chloride level in rainbow trout exposed to nickel.

Time of exposure (h)	Percent changes in serum chloride levels at various nickel concentrations (ppm)		
	0.8	2.8	4.64
0	100 ± 2.1[a](24)[b]	100 ± 6.2(22)	100 ± 12.4(12)
24-26	99 ± 2.1(6)	101 ± 4.0(5)	110 ± 6.9(4)
45-48	99 ± 2.2(6)	101 ± 3.1(6)	105 ± 4.4(3)
74			104 ± 7.0(3)
94-104		98 ± 2.8(6)	108 ± (1)
192	99 ± 3.9(6)	94 ± 2.7(6)	

[a]standard deviation
[b]number of observations

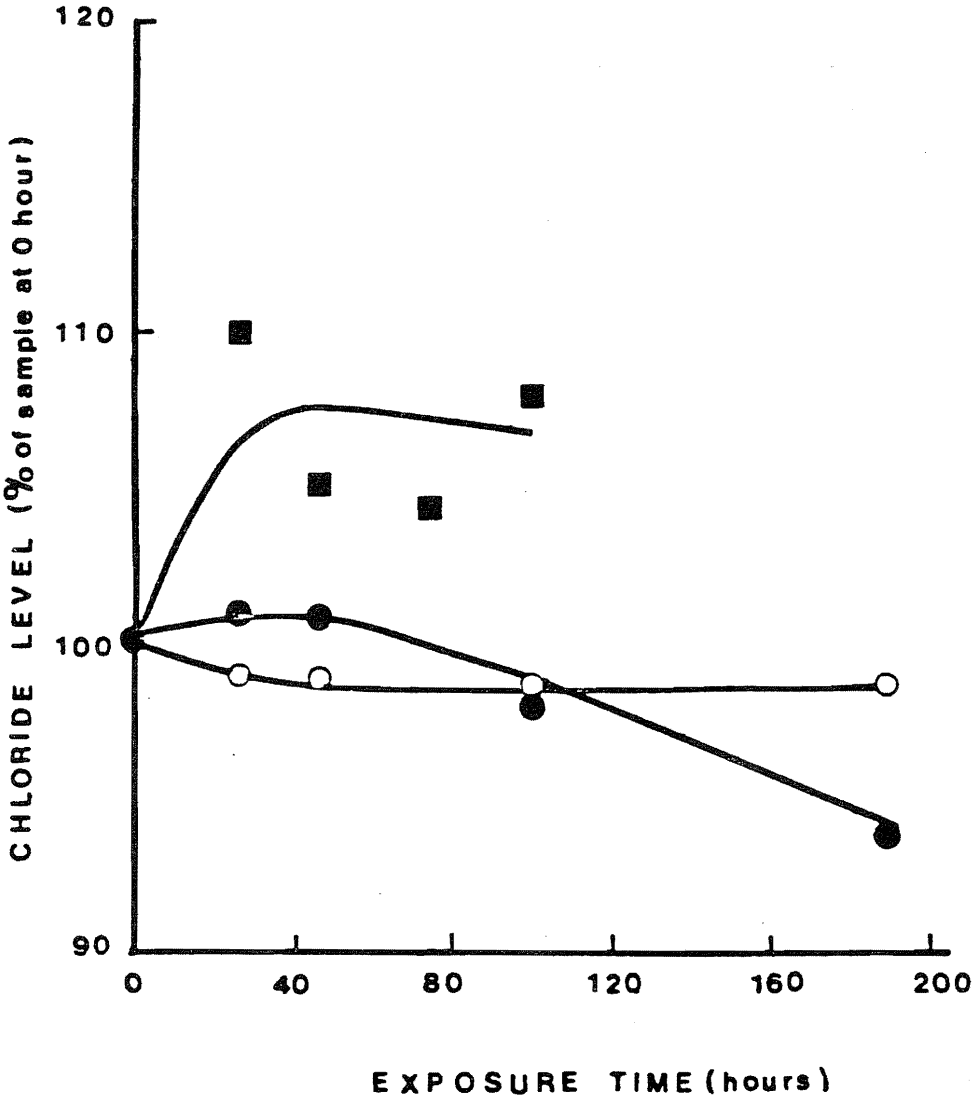

Figure 16. Changes in serum chloride level in rainbow trout upon their transfer to 0.8 (○), 2.8 (●), and 4.6 (■) ppm nickel.

Table 12. The lethal response of rainbow trout exposed to phenol for four days.

Mean concentration of phenol (ppm)	Percent mortality at 96 h
7.9	0.0
8.5	0.0
8.3	12.5
9.8	12.5
9.9	5.0
10.2	62.5
11.4	100.0
13.4	100.0
14.6	100.0

Dose-response function: $Y = -19.78 + 24.75 \log X$
Correlation coefficient: $r = 0.94$

Table 13. Lethal response of rainbow trout exposed to nickel solutions for four days.

Concentration of nickel (ppm ± SD)	Percent mortality at 96 h
21.8 ± 0.8	25.0
23.2 ± 1.2	12.5
26.4 ± 1.0	12.5
27.4 ± 1.3	62.5
30.5 ± 1.3	37.5
31.0 ± 1.2	87.5
34.0 ± 2.2	62.5
36.9 ± 1.8	87.5
40.9 ± 0.6	100.0

Dose-response function: $Y = -10.01 + 10.30 \log X$
Correlation coefficient: $r = 0.90$

Table 14. The lethal response of rainbow trout exposed to solutions of vanadium for four days.

Mean concentration of vanadium (ppm ± standard deviation)	Percent mortality at 96 h
7.6 ± 0.2	0.0
9.0 ± 0.5	25.0
11.2 ± 0.3	25.0
12.6 ± 0.3	75.0
15.2 ± 0.3	87.5
20.6 ± 0.6	100.0
24.2 ± 1.1	100.0
25.2 ± 0.9	100.0

Dose-response function $Y = -5.93 + 10.29 \log X$
Correlation coefficient: $r = 0.98$

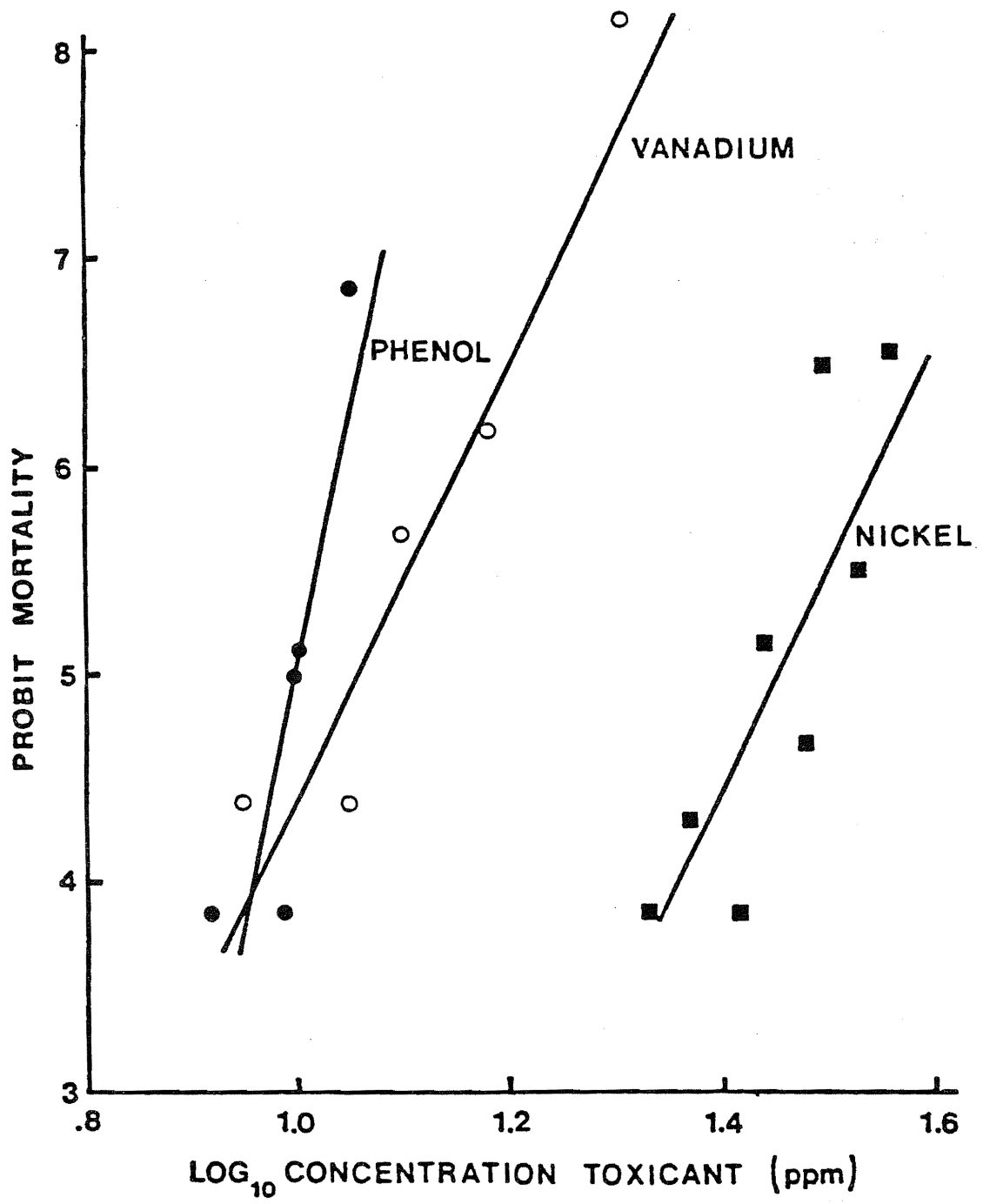

Figure 17. Dose-response regression lines for rainbow trout exposed to discrete solutions of nickel (■), vanadium (○), and phenol (●).

4.3.3 Toxicity of Binary Mixtures of Vanadium and Nickel

The regression coefficients of the linear functions which were fitted to the dose-response data of vanadium and nickel were not found to be significantly different according to Student's t-test ($p < 0.05$). This apparent parallelism between the quantal response curves supported the hypothesis that the two heavy metals may be similarly acting as constituents of lethal mixtures.

The results of subsequent tests based on the rationale that predicts strict addition (Section 2.1) between similarly acting substances are reported in Table 15. Figures 18 illustrates the probit regression for observed points which is compared to the line predicted for the binary mixture when one assumes strict addition. The two lines are not the same as the predicted line lies outside the 95% confidence-zone of the observed regression line. Consequently, the hypothesis that these apparently similarly acting compounds are strictly additive as lethal agents was rejected.

From the point of view that the similarity in slopes between the respective regression lines of vanadium and nickel may be coincidental, the response data for mixtures were re-evaluated in accordance with the model of independent action (Section 2.1). The latter theoretical model predicts that response addition occurs between toxic constituents of mixtures (Section 2.2). As in the former test, observed points do not fit the predicted line (Figure 19). Thus, neither hypothesis is supported. Furthermore, due to the proportions of each constituent within the particular mixtures studied, the regression lines which represent each hypothesis are almost identical. It was impossible from these data to distinguish between the two hypotheses of multiple toxicity. However, because the empirical data did not fit either model, it was deemed unnecessary to repeat the experiments.

Interactions may occur between similarly acting substances and result in either an enhanced response, i.e., supra-additive synergism, or a lessened response, i.e., infra-additive antagonism, to that predicted for strict addition (see isobologram, Figure 1). Figure 20 illustrates the effects of vanadium, at different

Table 15. The lethal response date for rainbow trout exposed to mixtures of vanadium and nickel for four days.

Concentration in equivalent vanadium (ppm)					Mortality			
Vanadium	Nickel	Mixture (assuming similar action)	Mixture (assuming independent action)	Observed mortality in each lot	Response (assuming similar action) observed probit[a]	Response (assuming similar action) predicted probit	Response (assuming independent action) observed probit[a]	Response (assuming independent action) predicted probit
6.8	2.49	9.29	6.80	1	3.91	4.03	3.89	2.64
8.5	3.34	11.84	8.50	2	4.27	5.11	4.21	3.63[b]
10.3	4.22	14.52	10.30	3	4.59	6.03	4.59	4.49[b]
12.8	5.19	17.99	13.54	2	4.95	6.98	4.97	5.71
14.0	6.40	20.40	16.00	6	5.13	7.55	5.19	6.46
16.3	6.52	22.82	18.22	4	5.30	8.05	5.34	7.04
19.0	7.16	26.16	21.08	7	5.52	8.66	5.56	7.69
20.0	9.10	29.10	23.01	6	5.66	9.13	5.69	8.08
27.0	11.84	38.84	28.09	7	6.12	10.42	5.97	8.98

[a] taken off fitted regression line
[b] within 95% confidence limits of observed probits
Dose-response assuming similar action: Y = 0.461 + 3.56 log X
Correlation coefficient: r = 0.90
Dose-response function assuming independent action: Y = 1.277 + 3.15 log X
Correlation coefficient: r = 0.91
Predicted dose-response function: Y = -5.93 + 10.29 log X
Correlation coefficient: r = 1.0

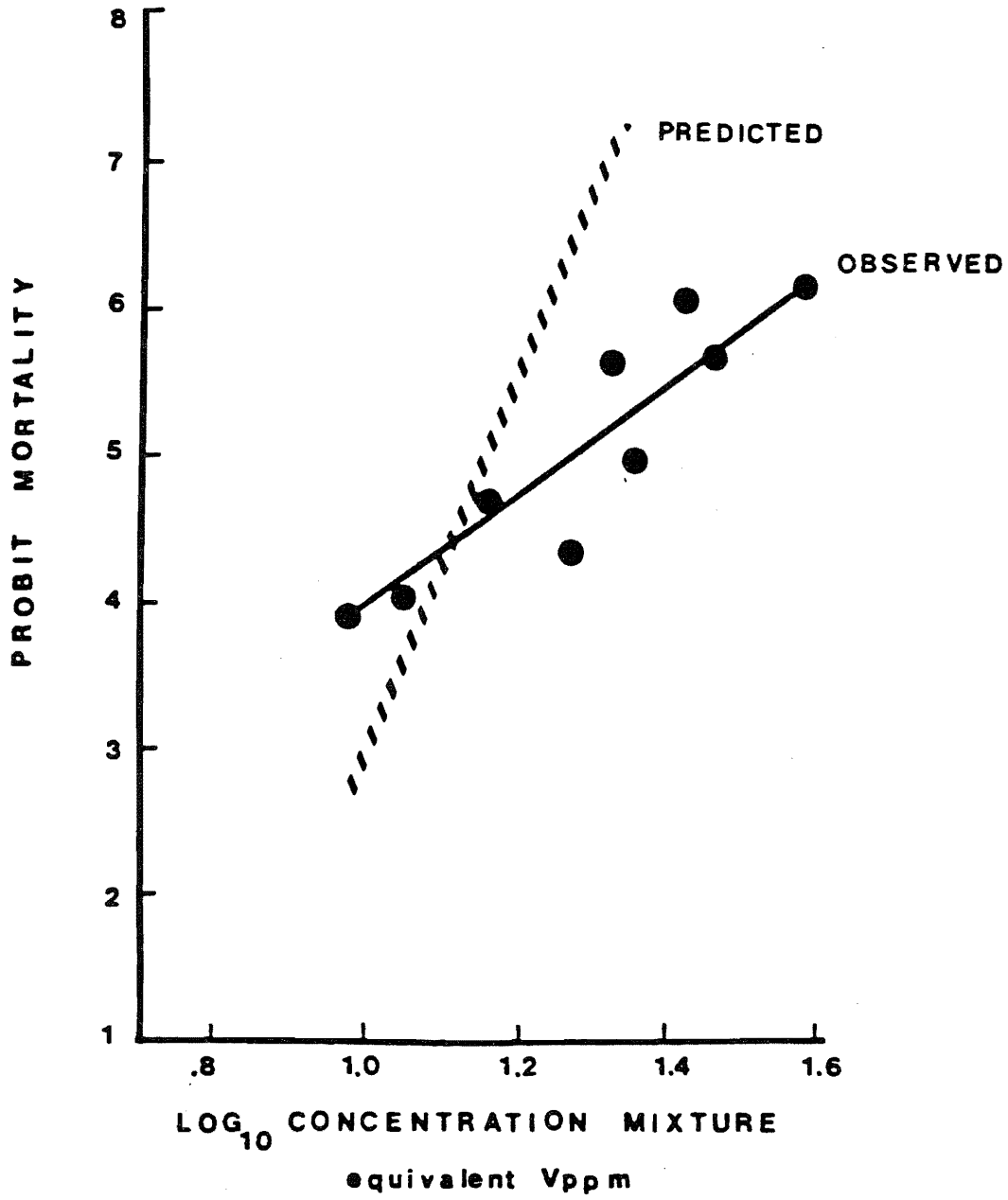

Figure 18. Predicted and observed dose-response regression lines for rainbow trout exposed to binary mixtures of vanadium and nickel which are assumed to be similarly acting.

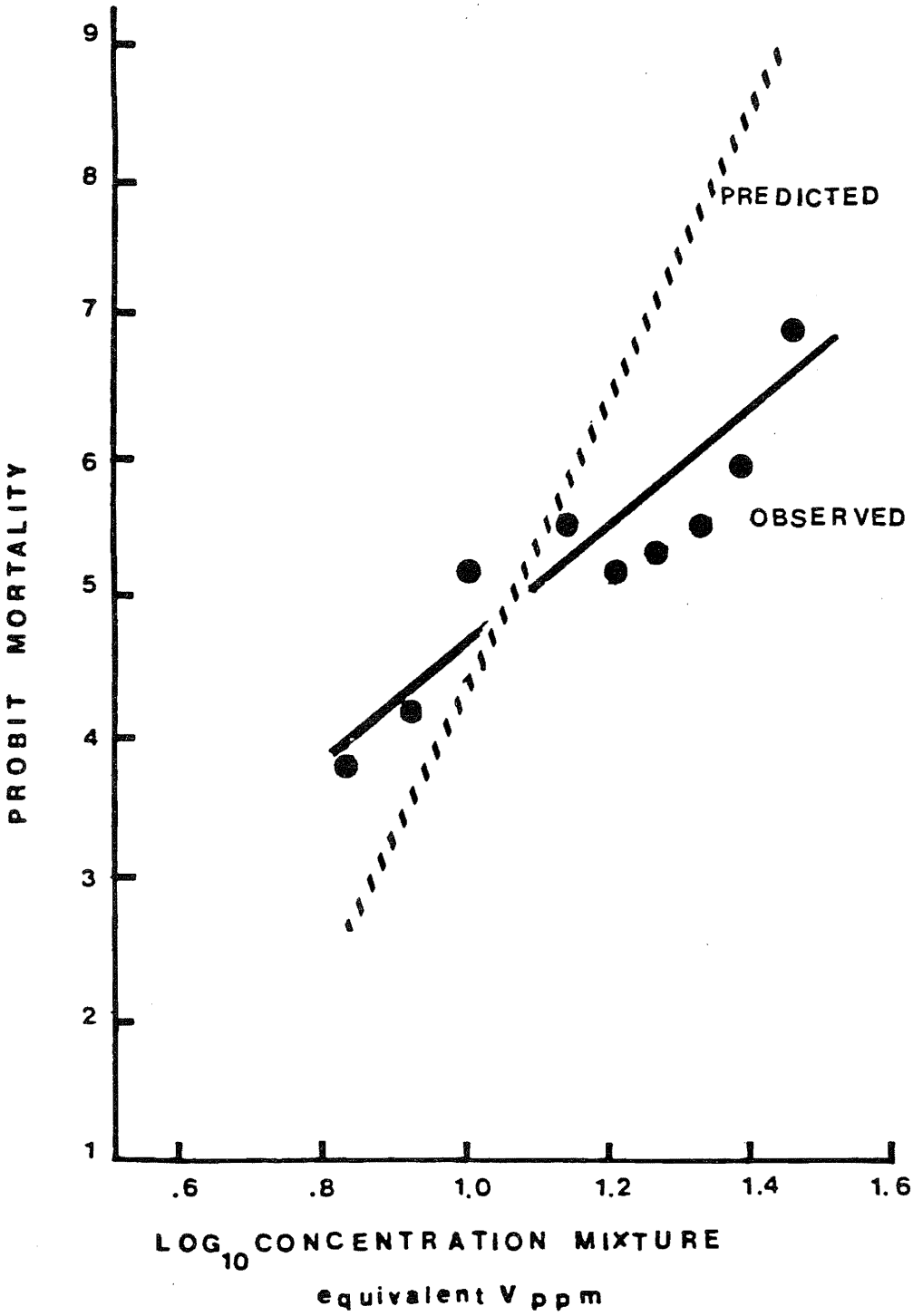

Figure 19. Predicted and observed dose-response regression lines for rainbow trout exposed to binary mixtures of vanadium and nickel which are assumed to be acting independently.

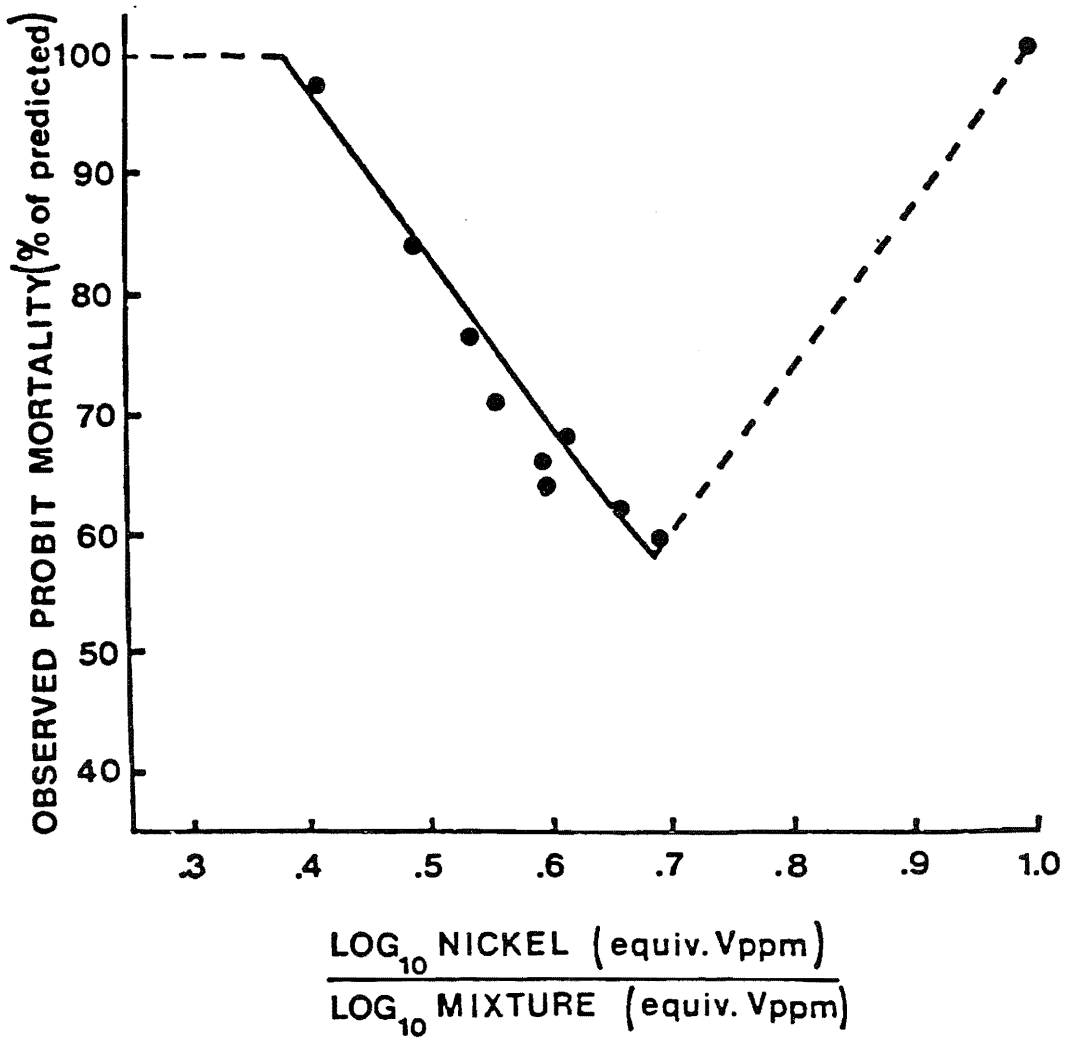

Figure 20. An isobol showing the decrease in lethal potency of nickel-vanadium mixtures from that predicted for strict addition. The antagonism increases as ratio of nickel to total concentration increases.

proportions within the binary mixtures, on the displacement of observed response from those predicted on the basis of strict addition. This graph shows that, as the proportion of vanadium is decreased relative to the combined concentration of the binary mixture, the lethal potency of the mixture becomes progressively less than that expected for strict addition. Within the concentration range studied, the constituents display infra-additive antagonism.

The antagonistic effect reaches a maximum at different proportions depending on the total concentration. For example, at a combined concentration equivalent to the LC_{50} of vanadium (in accordance with strict addition), the proportion of vanadium that produces a maximum effect is 54.3%. At a combined concentration equivalent to an LC_{10} (in accordance with strict addition), the maximum degree of antagonism is reached when vanadium is 49.8% of the mixture. Tests were not conducted to explore whether this antagonistic relationship between vanadium and nickel extends to sublethal effects. Hypothetically a decrease in the proportion of vanadium beyond that studied in this project would result at some point in a gradual lessening of antagonism (as depicted by the broken line in Figure 20).

4.3.4 Lethal Toxicity of Binary Mixtures of Vanadium and Phenol

A student's t-test indicated that the slope of the 96 h LC_{50} dose-response line for phenol was significantly different from those for vanadium and nickel ($p > 0.05$). In accordance with the proposed models of multiple toxicity (Sections 2.1 and 2.2), it was assumed that, in binary mixtures, independent action occurs between phenol and either metal.

Table 16 represents the observed lethal response data for juvenile rainbow trout. The experimental results are compared to the lethal responses which are predicted for response addition between independently acting substances. Furthermore, the table shows that for these data the predicted lethal effects for response addition are distinct from those predicted for strict addition.

Table 16. The lethal response data for rainbow trout exposed to mixtures of vanadium and phenol for four days.

Concentration in equivalent vanadium (ppm)				Mortality				
Vanadium	Phenol	Mixture (assuming similar action)	Mixture (assuming independent action)	Observed mortality in each lot	Response (assuming similar action) observed probit[a]	Response (assuming similar action) predicted probit	Response (assuming independent action) observed probit[a]	Response (assuming independent action) predicted probit
3.81	3.22	7.03	2.59	0	----	----	----	----
5.56	4.70	10.26	5.70	0	1.73	4.47	1.43	1.85
5.87	7.11	12.98	8.60	1	3.14	5.53	3.15	3.69[b]
5.48	10.65	16.13	11.75	2	4.55	6.50	4.58	5.08[b]
6.07	12.02	18.09	13.76	3	5.25	7.01	5.29	5.79[b]
6.53	15.71	22.24	17.70	8	6.52	7.93	6.40	6.91[b]
4.76	19.23	23.99	19.40	8	6.94	8.27	6.81	7.32[b]
6.67	20.76	24.73	22.25	8	7.08	8.41	7.42	7.93[b]
5.24	22.35	27.59	22.79	8	7.79	8.90[b]	7.52	8.04[b]

[a] taken off fitted regression line
[b] within 95% of confidence limits of observed probits
---- no response predicted or observed
Dose-response function assuming independent action: Y = -6.29 + 10.15 log X
Correlation coefficient: r = 0.90
Dose-response function assuming similar action: Y = -12.51 + 14.10 log X
Correlation coefficient: r = 0.91
Predicted dose-response function: Y = -5.93 + 1.29 log X
Correlation coefficient: r = 1.0

Figure 21 illustrates the observed points in relation to their fitted regression line which was derived by the weighted probit procedure of Finney (1971). Included in this figure are the theoretical regression lines predicted for response and strict addition. Tests for "goodness of fit" between observed and predicted co-ordinates for each multiple toxicity model support the assumption that vanadium and phenol act independently ($P > 0.05$). Thus, vanadium and phenol, within the range of relative proportions studied, contribute to the lethal toxicity of their mixtures as though each was acting as a pure solution.

4.3.5 Lethal Toxicity of Tertiary Mixtures of Vanadium, Nickel, and Phenol

Table 17 compares the observed lethal response data and the expected mortalities in lots of juvenile rainbow trout exposed to tertiary mixtures. In this test, the expected mortalities are computed on the assumption that vanadium and nickel are similarly acting and infra-additive and that phenol is independently acting and response additive.

The linear regressions derived for the observed and expected responses are shown in Figure 22. These lines are dissimilar as the predicted line lies outside the 95% confidence-zone of the observed regression line: thus the above assumption was negated.

Further analyses of the data indicated that, in those mixtures in which the total concentration is highest and in which phenol predominates, the observed responses approximate those predicted. However, as the total concentration of the mixtures declines and, coincidentally, the proportion of phenol decreases, the observed responses (one-tailed test) are significantly enhanced above predicted ($P > 0.05$). The relationship is illustrated in Figure 23. This figure shows that, as phenol's relative proportion decreased within the tertiary mixtures which in turn decreased in concentration, the observed mortality

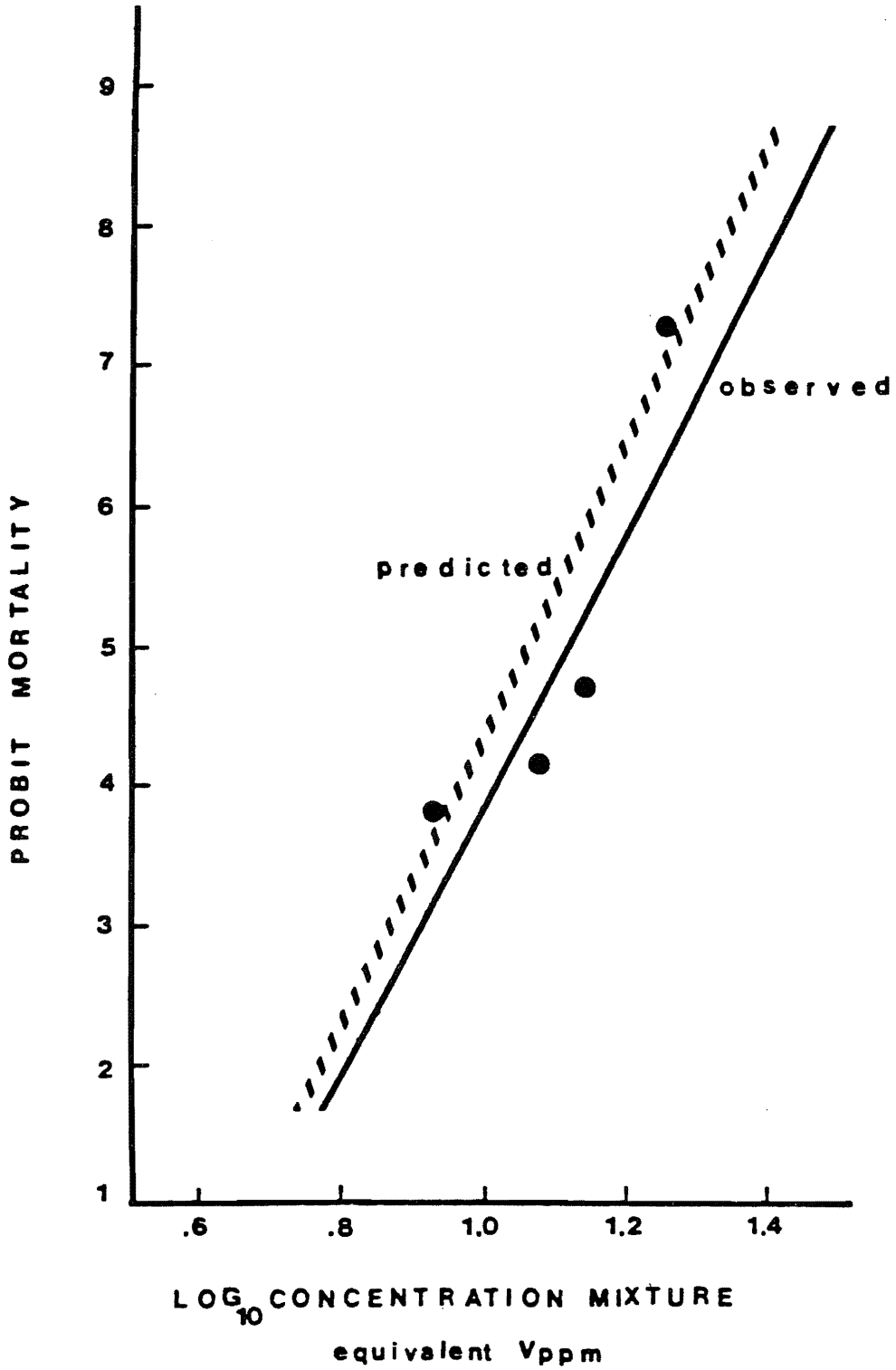

Figure 21. Predicted and observed dose-response regression lines for rainbow trout exposed to binary mixtures of vanadium and phenol which are assumed to be acting independently.

Table 17. The lethal response data for rainbow trout exposed to mixtures of vanadium, phenol, and nickel for four days, assuming nickel and vanadium to be antagonistic and phenol to be independent.

Concentration in equivalent vanadium (ppm)				Mortality		
Vanadium	Nickel	Phenol	Mixture[a]	Observed mortality in each lot	Observed probit[a]	Predicted probit[a]
1.2	2.33	0.45	4.57	1	4.31	0.86
4.1	4.55	1.95	6.20	5	4.81	2.22
3.2	4.75	0.89	6.22	2	4.81	2.23
5.5	5.10	3.48	6.87	6	5.00	2.68
4.2	6.41	4.84	8.12	5	5.27	3.43
4.8	6.29	7.29	10.75	5	5.73	4.68
5.0	6.77	9.10	13.50	6	6.11	5.70
5.9	8.75	11.96	17.79	8	6.57	6.93[b]
7.3	14.85	15.30	23.00	8	6.99	8.08[b]

[a] taken off fitted regression line
[b] within 95% confidence limits of observed probits
Observed dose-response function: $Y = 1.79 + 3.82 \log X$
Correlation coefficient: $r = 0.86$
Predicted dose-response function: $Y = 5.93 + 10.29 \log X$
Correlation coefficient: $r = 1.0$

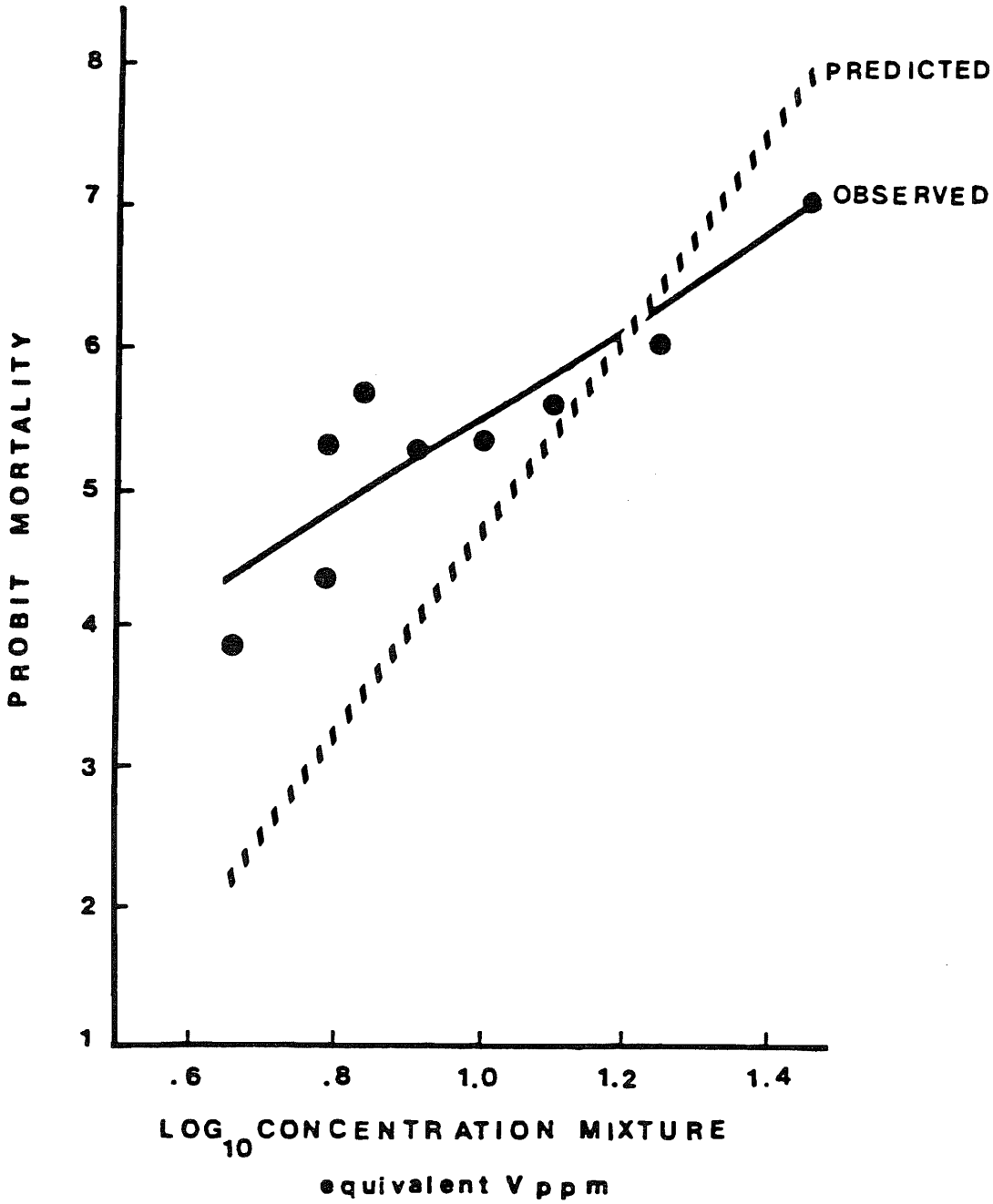

Figure 22. Predicted and observed dose-response regression lines for rainbow trout exposed to tertiary mixtures of vanadium, phenol, and nickel, assuming nickel and vanadium are similary acting and antagonistic and phenol is independently acting in the mixture.

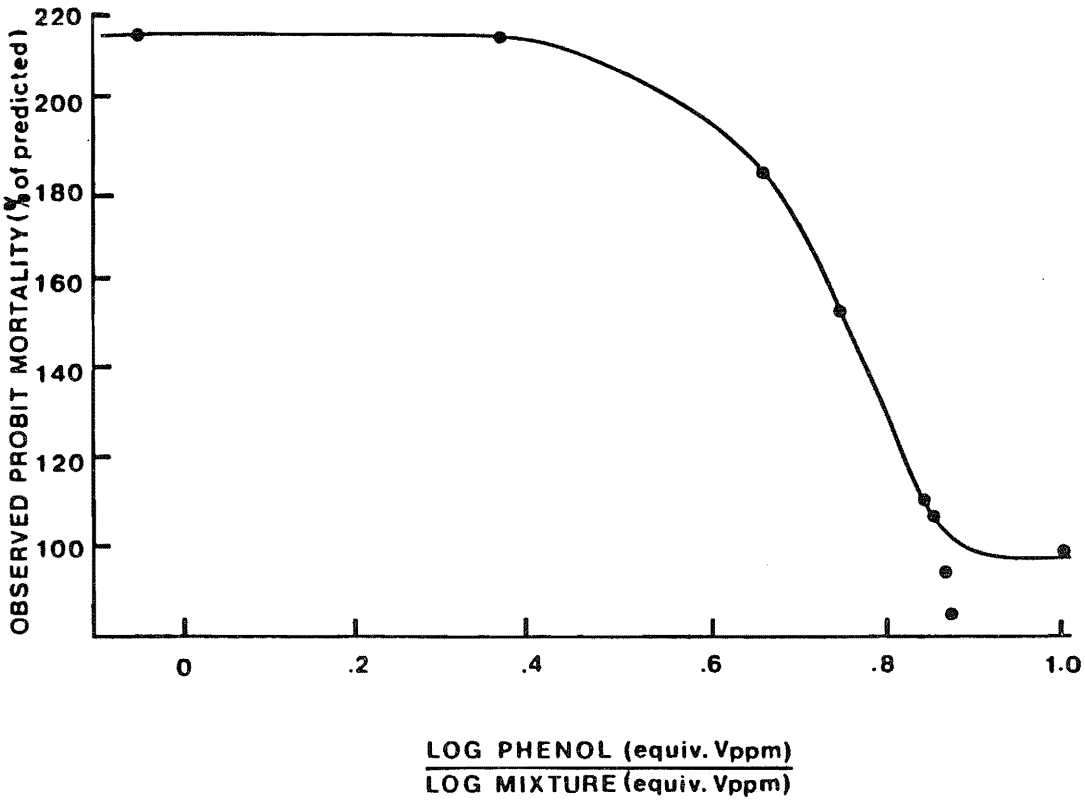

Figure 23. Isobol showing the increase beyond that predicted for the lethal potency of tertiary mixtures when the concentration of phenol relative to the total concentration of the mixture decreases.

progressively increased beyond predicted. Maximum mortality was reached when the concentration of phenol was approximately one half the total concentration of the tertiary mixtures. At this point the observed response was slightly greater than twice that predicted. With further decrease in the proportion of phenol and, concurrently, in the total concentration of the mixtures, there was no change in the degree of enhanced response within the range studied. Thus, within this range, the observed points are parallel to the expected co-ordinates. The results show that nickel and vanadium, in combination or perhaps singly, interact with phenol over a certain range of proportions and total concentration to cause a synergistic form of multiple toxicity.

5. DISCUSSION

5.1 LETHAL TOXICITY OF INDIVIDUAL SUBSTANCES

According to the following scale recommended by a joint committee of experts from IMCO/FAO/UNESCO/WHO (1969), phenol in pure solution is relatively toxic as a lethal agent. However, from the present studies, fish may develop resistance to this chemical by 24 h of exposure. This pattern of response is also characteristic of certain other phenolics, e.g., pentachlorophenol (Anderson and Weber 1975). The degree of "toxicity" was categorized in terms of the incipient lethal level (ILL) as follows:

1. "Very toxic" ILL below 1 ppm;
2. "Toxic" ILL between 1 and 100 ppm;
3. "Moderately toxic" ILL between 100 and 1000 ppm; and
4. "Slightly toxic" Ill between 1000 and 10 000 ppm.

Brown (1973) has rationalized that the death of fish as witnessed in ILL toxicity tests is the summative consequence of a number of physiological functions that fail. The curvilinear aspects of the ILL-toxicity curve that leads to an asymptote parallel to the time axis is interpreted as the progressive achievement of homeostasis in the test fish as the concentration decreases.

Nickel is judged to fall into the "toxic" category also even though an ILL was undeterminable within a 10 d period. Based on extrapolations from data collected in the laboratory during studies on nickel toxicity in other species of fish, *Brachydanio rerio* and *Poecilia reticulata*, the nickel ILL for trout should occur between 5 and 20 mg/L.

There was no indication of an ILL for vanadium in bioassays which extended over a 2 wk period (Figure 5). Furthermore, survivors of the 2 wk exposure period continued to die after having been placed in fresh water. Thus, the incipient lethal level of vanadium may occur below the milligram per litre criterion for "very toxic" substances as defined by the above scale.

The log time-log concentration plot for vanadium in these studies (Figure 5) was rectilinear throughout the 12 d period. Thus, according to Brown's (1973) rationale, above, there was no indication that any test fish were adapting to the presence of vanadium even at the lowest concentration of 2.8 mg/L.

This relationship suggests the hypothesis that vanadium is accumulating at least within the concentration range studied herein. Accumulation occurs when the rate of uptake exceeds the rate of elimination. Thus, even at very low ambient toxicant concentrations the total body burden might increase and lead to toxicity (Loomis 1974).

However, the accumulation of a toxicant may not lead to death immediately as certain contaminants are known to be ineffective while stored in fatty tissues but become poisonous when the fatty tissue is metabolized. Although some work has been done on accumulation of vanadium in fish (Sprague et al. 1978), total body burden may not be an indicator of toxicity (Spear and Anderson 1978). In order to predict the hazard of an accumulative toxicant, it is necessary to know not only the uptake and elimination rates (and thus the accumulation) but also possible rates of toxicant remobilization.

5.2 SUBLETHAL TOXICITY OF NICKEL

The threshold for the effects of nickel on trout osmo- and ionoregulation were calculated from shifts in serum osmolarity and serum chloride (Figures 14 and 15) to be between 0.8 and 2.8 mg/L. However, osmoregulation as an indicator of nickel toxicity may not be one of the more sensitive parameters of the primary effect of nickel because, even at 0.8 mg/L nickel, the serum protein level increased 20% above control values. Such a displacement in serum protein may be a reflection of either a displacement of water in response to nickel (a doubtful conclusion because osmolarity at this concentration changed only slightly) or a dysfunction in organ(s) tissue(s) (e.g., liver).

It is now recommended that application factors, estimating safe levels from lethal toxicity standards (e.g., 96 h LC_{50}), be replaced by life cycle and other studies that establish the maximum acceptable toxic concentration (MATC) criteria used in water quality management for the protection of aquatic organisms (Mount 1977). Nevertheless, if an application factor is computed for nickel based on short-term, sublethal studies on osmoregulation, the derived value is 0.03. This is close to the MATC derived application factor of 0.01 recommended as a universal safe level for nickel in the EPA red book. Because some effects were seen at 0.8 mg/L nickel on serum protein content, the recommendations would be to accept the lower application factor of 0.01 the LC_{50}.

5.3 SALINITY AND COMBINED SALINITY-NICKEL STRESSES

The rainbow trout, an euryhaline organism, is able to regulate the osmotic and ionic levels within body fluids in both fresh and seawater. The results of the tests, therefore, may not be applicable to those aquatic organisms which live in the watersheds around the Athabasca Oil Sands developments and are stenohaline (i.e., relatively intolerant of saline stress).

In freshwater, rainbow trout must oppose the influx of water by producing copious, hypo-osmotic urine; while, in saline environments, trout must oppose the loss of water and gain of ions (Schmidt-Nielsen 1974). As serum chloride and osmolarity (Figures 12 and 13) were significantly higher in rainbow trout upon exposure to 20 and 50% seawater, it might be supposed that the rainbow trout were not successful at osmo- and ionoregulation. However, Figure 24 demonstrates that rainbow trout were effective in maintaining a reasonably constant internal milieu. These adaptions, leading to osmotic and ionic stabilization, were achieved approximately 45 h after transfer to the saline water.

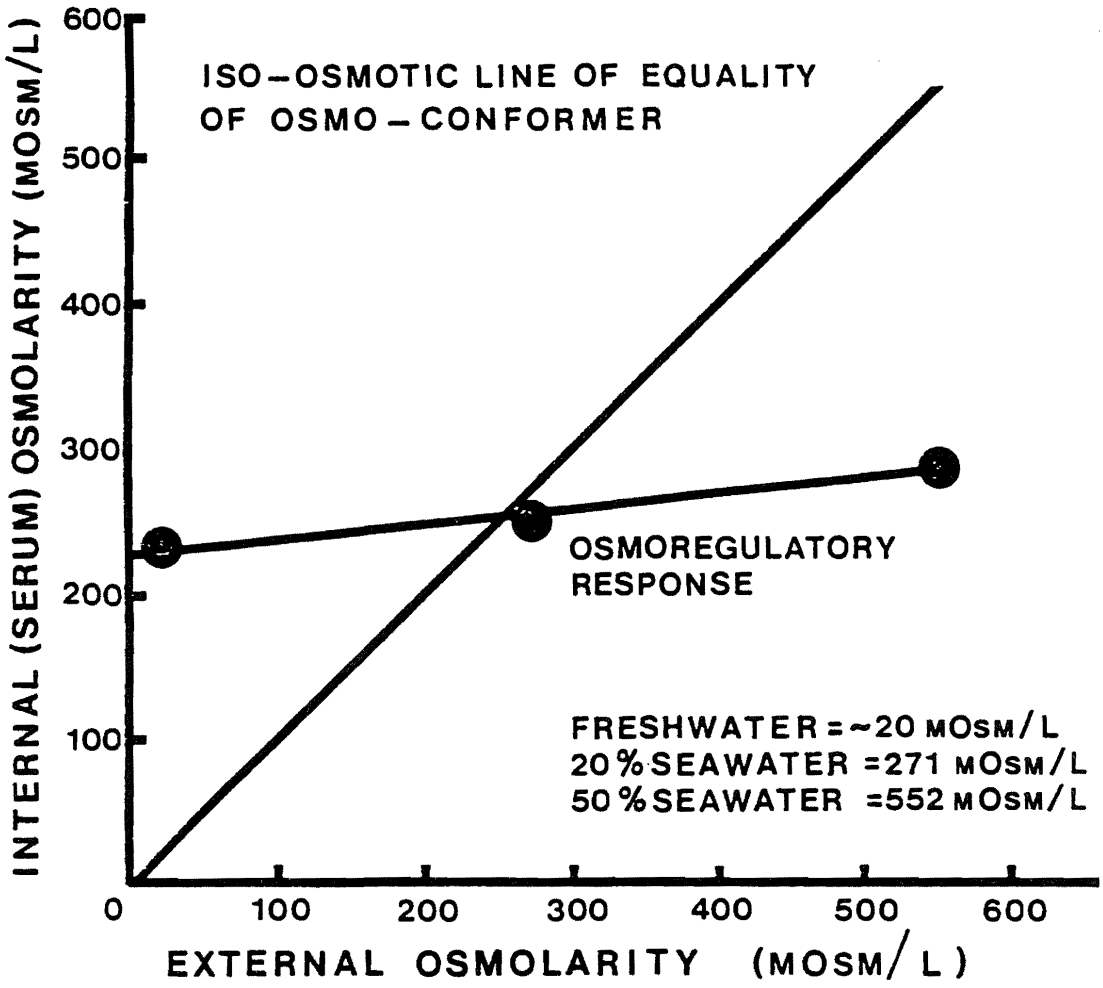

Figure 24. Serum osmolarity of rainbow trout relative to iso-osmotic levels for osmo-conformity in freshwater and 20 and 50% seawater.

Previous exposure of test fish to 5 ppm nickel had no effect on the ability of trout to adapt to subsequent exposure to salt water stress (Figures 12 and 13). This lack of interaction is all the more significant when it is realized that, in freshwater, nickel at 4.6 ppm caused an increase in both serum osmolarity and serum chloride levels. It would seem that these latter changes do not adversely affect the adaptive patterns that are initiated in fish upon exposure to salt-contaminated waters. Teleosts in marine environments have been known for many decades to have a high tolerance to nickel (Thomas 1915). Potts and Parry (1964) have reported that, in salt water environments, the teleost kidney removes divalent ions ($SO_2^{=}$, Mg^{++}, and Ca^{++}). Using such a transport mechanism, rainbow trout, in freshwater, may be excreting nickel via urine. This hypothetical explanation gains support from the study of Bryan (1971) who reported that aquatic organisms are able to remove heavy metals from the body through kidney excretions. If the presence of nickel stimulates urine output in exposed fish, then an increase in blood osmolarity and chloride level as seen in the fresh water experiments would be expected. These results are in contrast to those of McKim et al. (1970) who observed a fall in serum chloride and osmolarity in brook trout following chronic exposure to low levels of copper.

5.4 MULTIPLE TOXICITY

5.4.1 Nickel and Vanadium

Evidence gathered in independently conducted studies over several decades has shown certain heavy metals to be either strictly additive or supra-additive as constituents of lethal mixtures (Anderson and Weber 1975). Both patterns of multiple toxicity (Section 2) are considered highly hazardous because water quality standards based on criteria for discrete contaminants do not safeguard organisms against either phenomenon. Thus, there is concern about whether similar patterns of multiple

toxicity characterize mixtures of nickel and vanadium, the two metals in significant concentrations in the ore mined by the oil sands industry.

These initial studies on the lethal potency of binary mixtures of nickel and vanadium suggest that these particular heavy metals are antagonistic (Figure 20). The maximal reduction in response below that predicted for strict addition was 40%. The degree of infra-additive interactions increased as the proportion of nickel in the mixtures increased. However, mixtures in which vanadium predominated exhibited a lethal potency close to that predicted for strict addition.

As suggested in Section 1.3.2, nickel is released by the oil sands industry into the environment in amounts that are many fold less than vanadium. This difference in "loading rates" may mean that the prevalent heavy metal in binary mixtures encountered by organisms in receiving waters is vanadium. Where such a distribution does exist, the application of water quality standards which are based on the toxic unit principle is advised as follows (Esvelt et al. 1973):

$$T_{cr} = \frac{T_{cv}Q_1 + T_{cn}Q_2}{Qt} = 0.05TU$$

where TU = Toxic unit = 1

Tcr = total allowable relative concentration in receiving waters

Tc_v, Tc_n = $\frac{\text{concentration (in receiving waters)}}{LC_{50}}$ for each of vanadium and nickel respectively

0.05 = application factor

Qt = total environmental loading rate = sum of Q_1 for vanadium and Q_2 for nickel

Nickel and vanadium are not the only metals which are antagonistic. Selenium has been shown to lessen mercury toxicity in rats and fish (Burk et al. 1974; Kim 1977). As an antagonist, selenium is thought to increase, in the test organism's plasma,

the levels of selenoprotein to which mercury readily binds. The formation of HgSe-protein complexes reduces the actual dose of mercury which would otherwise reach critical target sites within an organism (Chen et al. 1974). The antagonism recorded for mixtures of nickel and vanadium in which constituents were in approximately equal proportions suggests that a similar interaction may exist to that between selenium and mercury.

Other investigations of the multiple toxicity of metal mixtures which contained nickel but not vanadium (e.g., nickel and copper) did not reveal antagonism (Weinstein and Anderson 1978). Consequently, it may be inferred that, in nickel-vanadium mixtures, vanadium is the essential component in the mechanism which leads to antagonism.

5.4.2 Phenol and Vanadium

This preliminary investigation of phenol-vanadium mixtures has suggested that, at the proportions studied, these pollutants act independently as lethal agents. Thus, a reasonable estimate of the lethal hazard which mixtures of vanadium and phenol represent to fish may be predicted from a knowledge of the toxicities of their respective pure solutions. Standards based on assessment of each pollutant, singly, should adequately protect trout and other less sensitive aquatic organisms against the lethal effects of their mixtures. However, the same conclusion may not hold for lethal mixtures in which the proportionality between constituents differs from those studied and/or for sublethal levels of vanadium and phenol mixtures.

5.4.3 Tertiary Mixtures

The results of the studies of mixtures containing nickel, vanadium, and phenol were not predicted by either strict or response addition models. The potency of the tertiary mixtures was significantly greater than that estimated from

studies of binary mixtures (Sections 5.4.1 and 5.4.2). It was assumed that some form of physiological interaction led to the observed supra-additive synergism. The synergism was particularly evident in tests where phenol did not represent the major constituent of the mixture.

Because vanadium and phenol, in binary solutions, are apparently independently acting (Section 5.4.2), the interaction of nickel with phenol was assumed to be the primary cause of the enhanced response observed in the tertiary mixtures. Only through further studies could this hypothesis be checked.

However, there is evidence through mammalian studies that certain heavy metals can enhance the toxicity of organic contaminants. Maines and Kappas (1977) and Tephley et al. (1978) have shown that various metals, including nickel, alter the liver's production of heme-proteins, such as the mixed-function exidases (P450 and P448). These effects may in turn lead to a lessening in the ability of the liver's microsomal system to metabolize organic poisons and thereby to reduce or eliminate their toxicity.

Thus, the preliminary results in this study suggest a reason for concern. It is recommended that further research be carried out to assess the significance of the apparent synergistic interactions between the heavy metals, nickel and/or vanadium and phenol.

6. CONCLUSIONS

1. Of the three toxicants studied, vanadium is the most potent lethal agent to juvenile rainbow trout. This criterion is based on 14 d exposure periods. Over brief periods of exposure, e.g., 48 h, phenol is the most toxic pollutant. All three toxicants are categorized as "reasonably toxic" to fish.

2. No incipient lethal level (ILL) was established for vanadium toxicity tests conducted over a 14 d period. This pattern suggests that vanadium may be an accumulative metal. The gill epithelium appeared to be a critical target site for vanadium.

3. Pre-exposure to sublethal solutions of nickel did not impair the capacity of the euryhaline fish, *Salmo gairdneri*, to adapt to salt-contaminated waters.

4. Nickel, at concentrations of 0.8 mg/L and above, altered significantly the blood parameters, serum protein, and osmolarity in fish held in freshwater.

5. An application factor of 0.01 X 96 h-LC_{50} (28 mg/L) is tentatively recommended for the establishment of safe levels for nickel.

6. Although phenol is a potent lethal agent (96 h-LC_{50}, 10 mg/L), those test organisms which survived of exposure to phenol continued to live seemingly unimpaired for the remainder of the 10 d test.

7. The potency of lethal mixtures of vanadium and nickel, where vanadium is the predominant constituent, are reasonably predicted by the principle of strict addition.

8. As the proportion of nickel increases relative to the total concentration of binary mixtures of nickel and vanadium, the potency of the mixture becomes progressively less than that predicted by the principle of strict addition. Thus, a form of antagonism arises from the interaction between these two metals at certain concentration ratios.

9. Phenol and vanadium act independently as lethal agents when present concurrently. These contaminants contribute to a common lethal response only when each is above its respective threshold to lethality.

10. Synergism is observed in tertiary mixtures containing vanadium, nickel, and phenol. The degree of synergism is a function of the relative proportion of phenol to the total concentration. The less phenol in a tertiary mixture, the more enhanced the effect. The interaction which leads to the synergism would appear to involve nickel and phenol.

11. Trace levels of nickel and vanadium were detected in whole tissues of rainbow trout with no known history of exposure to either of these metals. The mean levels were 1.45 and 0.298 μg/g respectively;

12. "Dry Ash" procedures for the determination of vanadium and nickel in tissues were developed for precision analyses by atomic absorption spectrophotometry.

7. IMPLICATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Water quality criteria for the protection of aquatic organisms are usually based on the assumption that a definable level of a pollutant may be assimilated by an ecosystem without harm to the endemic biota.

The application of such criteria to single contaminants may not be warranted where mixtures of pollutants are concerned. There exists the possibility that individual chemical constituents may interact within an organism to create an adverse reaction more insidious than defined for each substance when operating discretely. The focal question of this project was whether such adverse multiple toxicities were characteristic of the mixtures of three prominent petrogenic pollutants, vanadium, nickel, and phenol.

Data from these preliminary experiments suggest that vanadium may have some of the characteristics of selenium as an antagonist of the toxicity of another metal. However, the enhanced potency of the tertiary mixture containing both metals and phenol implies that either nickel or nickel in conjunction with vanadium interacts physiologically with phenol to the detriment of the target organism. In theory, this latter observation negates the application of water quality criteria based on scientific data derived for each substance individually.

Additional research is needed for the purpose of obtaining an explanation of the apparent synergism between nickel, vanadium, and phenol. This knowledge would be useful in estimating the hazard that these mixtures of chemical substances represent to aquatic biota.

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9. APPENDICES

9.1 ANALYTICAL TECHNIQUE FOR THE DETERMINATION OF NICKEL AND VANADIUM IN FISH TISSUE BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

The specifications of the atomic absorption spectrophotometer and general operating procedures are listed in Table 18. The normal levels of nickel in whole body tissue of rainbow trout were relatively high and were assayed by flame furnace technique. In contrast, graphite furnace methodology had to be used to detect the trace levels of vanadium in whole fish samples. All glassware was washed several times with detergent, soaked overnight in a 1:1 HCl/HNO_3 bath, and then rinsed at least three times with glass distilled water.

9.1.1 General Procedure for the Preparation of Aqueous-base Standard Solutions

Standard solutions for each heavy metal were prepared from their respective 1000.0 ± 0.2 ppm stock solutions supplied by Fisher Scientific Company Limited as Certified Fisher Standard Stock Solution. One hundred, 10, 1, and 0.1 ppm standard solutions were made up by diluting the appropriate standard solution in increments of 10 fold.

9.1.2 Procedure Initially Adopted for Testing of Rainbow Trout Base to Determine Content of Each Heavy Metal

9.1.2.1 Preparation of fish tissue. Unexposed whole fish were air dried to constant weight at 65 ± 5°C. The dried whole fish (approximately 50 to 100 g dried weight per batch) were macerated using a 20.5 cm test tube as a pestle and a polyethylene beaker as a mortar. The fish skin tends to form small tissue balls during this process. These were crushed immediately, because delay in this action permits hardening that in turn presents difficulties during the ashing procedure.

Table 18. Instrument specifications and general operations for nickel and vanadium analyses in rainbow trout tissues by atomic absorption spectrophotometry.

Specifications	Nickel	Vanadium
instrument	Perkin-Elmer Model 503	Perkin Elmer Model 503
radiation source	Nickel hollow cathode lamp	vanadium hollow cathode lamp
wavelength	232.0 nm	318 nm
slit width	0.2 nm	0.7 nm
background correction	D_2 beam corrector	D_2 beam corrector
readout	3-second integration	peak integration mode
furnace	4-inch single slot flame burner	HGA 2100 graphite furnace with ramp assembly
fuel	acetylene - 20 gauge units (71/min)	nitrogen - 40 gauge units (141/min)
oxidant	air - 40 gauge units (141/min)	
purge system		auto high temperature
drying temperature		125°C
drying time: HGA		30 sec
Ramp		10 sec
charring temperature		1400°C
ashing time: HGA		40 sec
Ramp		10 sec
atomizing temperature		2500°C
atomizing time: HGA		15 sec
Ramp		5 sec
recorder range		5 m
chart speed		20 mm/min

The finely macerated tissues, from various batches (a total of about 200 to 300 g is required), were thoroughly mixed together. The entire mass of tissue was dried to constant weight at 65 ± 5°C and then desiccated.

9.1.2.2 Ashing procedure. The following weights of macerated and dried fish tissue were weighed out in triplicate: 0.2000, 0.5000, 1.0000, 1.5000, 2.0000, 2.5000, 3.0000, and 4.0000 g. The samples were transferred to 50 mL beakers. A blank was carried through the preparatory ashing and acid extraction procedure. All samples, including the blank, were treated to the following ashing process: 30 min at 100°C, 30 min at 150°C, followed by an increase in temperature of 50°C every 30 min until 500°C was attained. The samples were ashed overnight (circa 14 h) at 500°C. Heavy smoke usually occurred between 250°to 300°C.

The samples were removed from the muffle furnace and allowed to cool to room temperature; 10 mL of concentrated HNO_3 were then added. Samples were heated carefully (without spattering) to a temperature close to the boiling point to digest the ash and to oxidize any carbon residue. Additional small amounts of concentrated HNO_3 were added as required to a final volume of 10 mL. The contents of the beaker were carefully evaporated just to the point of dryness, without overheating or baking. Concentrated HCl in amounts of 0.50 ± 0.01 mL and 0.25 ± 0.01 mL for nickel and vanadium determinations, respectively, was pipetted into the beaker and swirled to mix. Water at a volume of 19.50 ± 0.02 mL and 9.75 mL, for nickel or vanadium determinations, respectively, was added to the beaker and mixed.

9.1.3 Nickel Determinations

9.1.3.1 Base tissue samples: standard curve and data. a set of working standards prepared for this test gave the following results:

Nickel (ppm)	Average Absorbance[a]	Standard Deviation
0.000	0.000	± 0.000
0.100 ± 0.002	0.004	± 0.001
0.250 ± 0.003	0.010	± 0.000
0.500 ± 0.003	0.020	± 0.000
1.000 ± 0.004	0.040	± 0.000
1.500 ± 0.020	0.059	± 0.000
2.000 ± 0.020	0.079	± 0.001

[a]average of five 3-second integration readings.

The linear regression (0 to 2) for the above data can be expressed in the form of:

absorbance = b + m Concentration (ppm)

is: a = 0.0001 + 0.03943 X

where a = absorbance

x = Concentration nickel (ppm)

The correlation coefficient (r) was 0.9997. The sensitivity (the concentration of nickel at the lowest detectable absorbance) was 0.111 ppm nickel, i.e., 2.2 µg Ni in the 20 mL sample analyzed. The linear range was 0.111 to 5 ppm. The values of nickel calculated by this function were subjected to a maximum deviation of ± 0.003 ppm.

Blank and fish tissue samples, weighing 1, 1.5, 2, 2.5, 3, and 4 g, each in triplicate, were treated and the nickel content was determined as above and is presented below.

Fish weight (g)	Ni content per sample (μg)	Base nickel content in fish (ppm)
1	1.34 ± 0.30	1.34 ± 0.30
1.5	2.36 ± 0.30	1.57 ± 0.28
2	2.53 ± 0.51	1.27 ± 0.26
2.5	3.21 ± 0.58	1.28 ± 0.23
3	3.89 ± 0.30	1.30 ± 0.12
4	4.56 ± 0.0	1.14 ± 0.0

However, in the fish samples weighing 2 g or less, five contained levels of nickel below the level of detectability. Therefore, the base nickel content of fish tissue, determined on the basis of the remaining 13 values, was 1.33 ± 0.21 ppm. Thus, the minimum amount of fish tissue in which base levels of nickel can be determined was about 2 g dry weight. Where smaller quantities of tissue were to be analyzed, graphite furnace procedures had to be employed.

9.1.3.2 Spiked fish tissue samples: standard curve and data. A standard curve was prepared as in Section 9.1.3.1 except that standards included 3, 3.5, 4, and 5 ppm samples. The linear regression for optical density vs. concentration nickel is the following:

$$a = 0.0003 + 0.0369\ x$$

where a is absorbance; x is nickel concentration(s) (ppm). The correlation coefficient (r) is 0.99991 and the sensitivity is 0.119 ppm nickel (2.38 μg Ni in 20 mL sample analyzed). The linear range is from 0.119 to 5 ppm nickel.

9.1.3.3 Recovery of nickel from spiked fish samples. Thirty samples of macerated and dried fish samples were weighed and spiked with nickel. The tissue weight range was from 1.1014 to 1.57 g and the average weight and standard deviation were 1.4481 and 0.0912. Each sample was spiked with either 0, 15 ± 0.2,

30 ± 0.3, 50 ± 0.3, 75 ± 0.4, or 100 ± 0.4 µg Ni. There were five replicates of each treatment. Subsequently, each sample was ashed as described in Section 9.1. and the nickel content was determined. The 95% probability criterion was used to reject any values that were spurious.

For the unspiked samples, the level of nickel in the tissue samples analyzed (circa 1 to 2 g) was below the sensitivity of the technique and, therefore, the base levels were determined by extrapolation from the spiked samples (Table 19).

As there was a loss of nickel from those samples spiked with 100 µg nickel, these values were not included in the calculations of base levels of nickel. Thus, the nickel content in dried fish tissues, calculated from spiked fish tissues, was found to be 1.54 ± 0.49 ppm. This value is not significantly different ($P < 0.05$) from the value obtained from the unspiked samples. These two values were combined and the nickel content of dried rainbow trout tissue not exposed to experimentally high values of nickel was calculated to be 1.45 ± 0.40 ppm.

The above base line value was used to calculate the percent recovery which was found to be virtually 100% within the spiking range up to 75 µg nickel, dropping to 98% at a spike level of 100 µg nickel.

9.1.3.4 Recovery of nickel from aliquots of spiked fish base samples. Two sets of dried fish tissues (2 g) were spiked with either 70 or 90 µg nickel. Prior to analysis, a 10 mL aliquot of each test solution was tested; the other 10 mL were diluted to 20 mL with 2.5% HCl and then tested. The percentages of nickel recovery for each case were calculated utilizing a base line value for the nickel content of rainbow trout of 1.45 ppm and are presented below.

Table 19. Determination of nickel in spiked samples of fish tissue.

Added Ni	Total Ni measured in sample (µg)[a]	Tissue Ni content[b] corrected for spike (ppm)	% recovery
15 ± 0.2(2)	17.34 ± 0.36[c]	1.58 ± 0.03[d]	101.05 ± 0.17[e]
30 ± 0.3(4)	32.38 ± 0.36	1.65 ± 0.17	100.29 ± 1.20
50 ± 0.3(5)	52.57 ± 0.86	1.82 ± 0.62	101.00 ± 0.169
75 ± 0.4(5)	76.54 ± 0.59	1.15 ± 0.42	99.48 ± 0.77
100 ± 0.4(5)	99.75 ± 0.82	-0.170 ± 0.54	97.62 ± 0.79

[a]total Ni measured (µg x 100)
[b]spike (µg) + 1.45 µg Ni/g fish tissue
[c,d,e]standard deviation

µg Ni added	treated	% recovery
70	diluted	105.9
70		104.3
90	diluted	100.3
90		99.2

Thus, the dilution of aliquots of tissues containing high levels of Ni did not appear to introduce additional variability.

9.1.3.5 Conclusions. It is concluded that the drying, ashing, and extraction procedure as described herein is efficient in the analysis of dried rainbow tissue for nickel. Where tissue samples contain low levels of nickel, the samples can be spiked. When tissue samples contain high levels of nickel, i.e., above the linear range, aliquots can be taken and diluted. The base line value of nickel in rainbow trout tissues is 1.45 ± 0.40 ppm.

9.1.4 Vanadium

9.1.4.1 Determination of the standard curve for vanadium.

Vanadium (ppm)	Average Absorbance[a]
0.000	0.000
0.025	0.008 ± 0.002
0.050	0.015 ± 0.002
0.100	0.028 ± 0.000
0.250	0.066 ± 0.001
0.500	0.130 ± 0.003
00.750	0.201 ± 0.005

[a]average of 5, peak integrations

The linear regression of absorbance over vanadium concentration is:

$$A = 0.0007 + 0.2643\ X$$

where A = absorbance

X = vanadium concentration (ppm)

The correlation coefficient (r) is 0.99977. The sensitivity is 0.016 ppm vanadium, i.e., 0.16 µg vanadium in the 10 mL sample analyzed. The values of vanadium calculated by this function are subject to a maximum error of ± 0.0047 ppm.

A blank and 19 fish tissue samples weighing from 0.2043 to 2.9737 g were treated and the vanadium content was determined as above. In the fish samples weighing 0.501 g or less (4 samples), the vanadium content was below detectability; therefore, the base vanadium content was calculated on the basis of the remaining 14 values and was found to be 0.27 ± 0.06 ppm.

9.1.4.2 Spiked fish tissue samples: standard curve and data. A standard curve was prepared for vanadium as in Section 9.1.4.1 except that the standards consisted of 0.05, 0.1, 0.25, 0.5, 0.75, and 1.00 ppm samples. The linear regression of optical density on vanadium concentration was the following:

$$a = 0.0006 + 0.27008\ x$$

where a = absorbancy

x = vanadium concentration (ppm)

The correlation coefficient (r) was 0.9999 and the sensitivity was 0.016 ppm vanadium (0.16 µg nickel in the 10 mL sample analyzed).

Thirty samples of macerated and dried fish samples were weighed and spiked with vanadium. The tissue weights varied from 0.9993 to 1.0212 g and the mean weight and standard deviation was 1.0025 ± 0.0213 g.

Each sample was spiked with either 0, 1.5 ± 0.03, 3 ± 0.03, 4.5 ± 0.04, 6 ± 0.04, or 7.5 ± 0.05 µg vanadium.

Subsequent to spiking, each sample was placed in the oven and heated at just under 100°C, until the liquid spike volume had dried out, and treated as described in Section 9.1.4. A blank with no spike was carried through the same procedure.

For the unspiked samples, the base level of vanadium in rainbow trout tissue was determined to be 0.31 ± 0.029 ppm. When these are combined with the 14 values obtained in Section 9.1.4, a value of 0.278 ± 0.06 ppm was obtained for the average vanadium content of dried (65 ± 5°C) rainbow trout unexposed to vanadium. Table 20 indicates the base-line levels of vanadium in the spiked tissue samples as well as the percentage recovery.

Thus, the vanadium content in dried fish tissues, calculated from spiked fish tissues, was found to be 0.31 ± 0.179 ppm. The mean vanadium content was not significantly different from that determined for the unspiked samples; however, the standard deviation is greater. The vanadium content of dried rainbow trout tissue not exposed to experimentally high values of vanadium calculated from all 49 samples was 0.298 ± 0.14 ppm. Utilizing this base level to calculate the percent recovery, the mean percent recovery was found to be 99.8 ± 4.0.

9.1.4.3 Recovery of vanadium from aliquots of spiked fish base samples. Two sets of dried fish tissues (1 g) were spiked with either 6 or 7.5 µg vanadium. Prior to analysis, a 5 mL aliquot of each test solution was tested; the other 5 mL were diluted to 10 mL with 2.5% HCl and then analyzed. The percentages of vanadium recovery for each case were calculated utilizing a base-line value for the vanadium content of rainbow trout of 0.298 ppm and are presented below:

µg added	treatment	% recovery
6.0	diluted	97.5
6.0		102.6
7.5	diluted	98.7
7.5		103.2

Table 20. Determination of vanadium in spiked samples of fish tissue.

Added vanadium	Total vanadium measured in sample (µg)	Tissue vanadium content corrected for spike (ppm)[b]	% Recovery[a]
15.0 ± 0.03 (5)	1.796 ± 0.099	0.295 ± 0.099	98.3 ± 5.1
3.0 ± 0.03 (5)	3.206 ± 0.099	0.208 ± 0.102	97.2 ± 3.0
4.5 ± 0.04 (5)	4.960 ± 0.206	0.451 ± 0.198	103.3 ± 4.2
6.0 ± 0.04 (5)	6.314 ± 0.275	0.322 ± 0.278	100.3 ± 4.5
7.5 ± 0.05 (5)	7.790 ± 0.129	0.289 ± 0.129	99.9 ± 1.7

[a] $\frac{\text{total vanadium measured (µg)}}{\text{spike (µg)}} \times 100$

[b] + 0.298 µg V/g fish tissue

Thus, although the recovery of vanadium from aliquoted samples was somewhat decreased, the recovery percent values are within the tolerance limits from vanadium recovery on spiked samples.

9.1.4.4 Conclusions. The vanadium content of dried rainbow trout, not exposed to experimentally high vanadium levels, was determined from 44 spiked and unspiked samples to be 0.298 ± 0.14 µg/g.

The recovery of vanadium from 25 1g samples of dried rainbow trout tissues which had been spiked within a range of up to 7.5 µg vanadium was found to be 99.8 ± 4%. Based on this recovery percent, it is concluded that the procedures described above are efficient in the analysis of the vanadium content of dried rainbow trout tissues.

When fish tissues contain vanadium at a concentration above the linear range, aliquots can be taken and diluted.

9.1.5 Step by Step Procedure Recommended for Preparation of Rainbow Trout Tissue Relative to the Determination of Nickel Content

1. Dry the whole fish specimens to constant weight at 65 ± 5°C.
2. Macerate the entire group of dried test fish (not more than 50 to 100 g per batch) using the technique described in Section 9.1.2.1.
3. Mix together well all batches of macerated tissue from (2) above and again dry to constant weight at 65 ± 5°C. Store in a good desiccator.
4. Weigh out the required sample weights in at least quintuplicate. Not less than 2.0 g should be weighed for each sample. Place each sample in a clean 50 mL beaker. Cover the beaker with a watchglass. Run a blank determination simultaneously.
5. Ash under the conditions described in Section 9.1.2.2.

6. Remove the beakers from the muffle furnace and allow to cool to room temperature. Add 10 mL of concentrated HNO_3. Heat without spattering to close to the boiling point to digest the ash and to oxidize, as well as possible, any carbon residue. Be sure that any ash spattered on the watchglass inside surface is included. Add small amounts of concentrated HCO_3 where required to maintain the original 10 mL volume.
7. Evaporate carefully until the beaker contents are just dry. Do not overheat or bake.
8. Pipette 0.50 ± 0.01 mL of concentrated HCl over the inside surface of the watchglass and into the beaker. Mix well.
9. Pipette 19.50 ± 0.02 mL of water over the inside of watchglass and into the beaker. Mix well.
10. Transfer to well-sealed plastic bottles of not more than 25 mL capacity and deliver to the atomic absorption test laboratory. All bottles are to be marked with the actual weight of dried rainbow trout tissue contained in the 20 mL sample volume.
11. Results will be reported in micrograms of nickel per 20 mL for each sample submitted, and this will represent the total nickel in micrograms for the fish tissue dried weight taken. Corrections relative to any contribution of nickel from the dried fish base weight used should be made by the person who originated the samples, and should be based for rainbow trout on the value found by this survey of 1.45 ± 0.40 µg Ni/g dried fish tissue. Where other than rainbow trout is involved, a survey similar to the present will have to be conducted to establish the value for the nickel content of unexposed dried base fish tissue.

12. All calculations for the results reported in (11) above will be based on aqueous-base working standards as prepared for sample series under investigation.

9.1.6 Step by Step Procedure Recommended for the Preparation of Rainbow Trout Tissue Relative to the Determination of Vanadium Content

1. Dry the whole fish tissue specimens to constant weight at 65 ± 5°C.
2. Macerate the entire group of dried test fish (not more than 50 to 100 g per batch) using the technique described in Section 9.1.2.1.
3. Mix well together all batches of macerated tissue from (2) above and again dry to constant weight at 65 ± 5°C. Store in a good desiccator.
4. Weigh out the required sample weights in at least quintuplicate into 50 mL beakers. Not less than 1.0 g should be weighed for each sample. Cover the beakers with watchglasses. Carry simultaneously a blank determination.
5. Ash under the conditions described in Section 9.1.2.2.
6. Remove the beakers from the muffle furnace and cool to room temperature. Add 10 mL of concentrated HNO_3. Heat without spattering to close to the boiling point to digest the ash and to oxidize as well as possible any carbon residue. Be sure that any ash spattered on the watchglass inside surface is included. Add small amounts of concentrated HNO_3 if required to maintain the original 10 mL volume.
7. Evaporate carefully until the beaker contents are just dry. Do not overheat or bake.
8. Pipette 0.25 ± 0.01 mL of concentrated HCl over the inside surface of the watchglass and into the beaker. Swirl carefully to mix.

9. Pipette 9.75 ± 0.02 mL of water over the inside surface of the watchglass and into the beaker. Mix well.
10. Transfer to well-sealed plastic bottles of not more than 25 mL capacity and deliver to the atomic absorption test laboratory. All bottles are to be marked with the actual weight of dried rainbow trout tissue contained in the 10 mL of sample volume.
11. Results will be reported in micrograms of vanadium per 10 mL for each sample submitted, and this will represent the total vanadium in micrograms for the fish tissue dried weight taken. Corrections relative to any contribution of vanadium from the dried fish base should be made by the person who originated the samples, and should be based on the value, found for rainbow trout by this survey of 0.298 ± 0.14 µg V/g dried fish tissue. Where other fish than rainbow trout is involved, a survey similar to the present will have to be conducted to establish the value for the vanadium content of unexposed base fish tissue.
12. All calculations for the results reported in (11) above will be based on aqueous-base working standards as prepared for the sample series under investigation.

9.1.7 Modifications to Technique (Section 9.1.6) for the Determination of Vanadium in Flagfish Tissue.

1. Amounts of fish tissue, selected on the basis of expected vanadium content, were homogenized and dried according to Section 9.1.2.1.
2. Dried samples were ashed according to Section 9.1.2.2, except that the samples, upon removal from the muffle furnace, were taken up with 5.00 ± 0.01 mL 3% HCl.

3. Standards of 50, 100, 200, 400, 600, 800, 1000, and 1500 ppb vanadium were prepared in 3% HCl.
4. Anticipated limits of detection for vanadium (ppb) in dried fish tissue of various quantities were as follows:

Tissue sample (g dried weight)	Injected volume 20 µL	50 µL	100 µL
0.02	3.2 ppb	1.3 ppb	0.6 ppb
0.05	1.3 ppb	0.5 ppb	0.3 ppb

Sample size (g)	20 µL	50 µL	100 µL
0.1	0.6 ppb	0.3 ppb	0.1 ppb
0.2	0.3 ppb	0.1 ppb	0.06 ppb
0.3	0.2 ppb	0.08 ppb	0.04 ppb

5. Each sample was tested five times to yield an average value and standard deviation.
6. Selected samples were spiked according to Section 9.1.4.2 to determine linearity of additive response in absorbance and recovery.
7. The program for graphite furnace was as follows: drying temperature--100°C; charring temperature--1500°C; and atomizing temperature--2800°C. The 2800°C atomizing temperature is recommended over the original 2500°C indicated in Table 20 to avoid residues in graphite rod.

10. AOSERP RESEARCH REPORTS

1.		AOSERP First Annual Report, 1975
2.	AF 4.1.1	Walleye and Goldeye Fisheries Investigations in the Peace-Athabasca Delta--1975
3.	HE 1.1.1	Structure of a Traditional Baseline Data System
4.	VE 2.2	A Preliminary Vegetation Survey of the Alberta Oil Sands Environmental Research Program Study Area
5.	HY 3.1	The Evaluation of Wastewaters from an Oil Sand Extraction Plant
6.		Housing for the North--The Stackwall System
7.	AF 3.1.1	A Synopsis of the Physical and Biological Limnology and Fisheries Programs whithin the Alberta Oil Sands Area
8.	AF 1.2.1	The Impact of Saline Waters upon Freshwater Biota (A Literature Review and Bibliography)
9.	ME 3.3	Preliminary Investigations into the Magnitude of Fog Occurrence and Associated Problems in the Oil Sands Area
10.	HE 2.1	Development of a Research Design Related to Archaeological Studies in the Athabasca Oil Sands Area
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12.	ME 1.7	Very High Resolution Meteorological Satellite Study of Oil Sands Weather: "A Feasibility Study"
13.	ME 2.3.1	Plume Dispersion Measurements from an Oil Sands Extraction Plant, March 1976
14.		
15.	ME 3.4	A Climatology of Low Level Air Trajectories in the Alberta Oil Sands Area
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21.		AOSERP Second Annual Report, 1976-77
22.		
23.	AF 1.1.2	Acute Lethality of Mine Depressurization Water on Trout Perch and Rainbow Trout
24.	ME 1.5.2	Air System Winter Field Study in the AOSERP Study Area, February 1977.
25.	ME 3.5.1	Review of Pollutant Transformation Processes Relevant to the Alberta Oil Sands Area

26.	AF 4.5.1	Interim Report on an Intensive Study of the Fish Fauna of the Muskeg River Watershed of Northeastern Alberta
27.	ME 1.5.1	Meteorology and Air Quality Winter Field Study in the AOSERP Study Area, March 1976
28.	VE 2.1	Interim Report on a Soils Inventory in the Athabasca Oil Sands Area
29.	ME 2.2	An Inventory System for Atmospheric Emissions in the AOSERP Study Area
30.	ME 2.1	Ambient Air Quality in the AOSERP Study Area, 1977
31.	VE 2.3	Ecological Habitat Mapping of the AOSERP Study Area: Phase I
32.		AOSERP Third Annual Report, 1977-78
33.	TF 1.2	Relationships Between Habitats, Forages, and Carrying Capacity of Moose Range in northern Alberta. Part I: Moose Preferences for Habitat Strata and Forages.
34.	HY 2.4	Heavy Metals in Bottom Sediments of the Mainstem Athabasca River System in the AOSERP Study Area
35.	AF 4.9.1	The Effects of Sedimentation on the Aquatic Biota
36.	AF 4.8.1	Fall Fisheries Investigations in the Athabasca and Clearwater Rivers Upstream of Fort McMurray: Volume I
37.	HE 2.2.2	Community Studies: Fort McMurray, Anzac, Fort MacKay
38.	VE 7.1.1	Techniques for the Control of Small Mammals: A Review
39.	ME 1.0	The Climatology of the Alberta Oil Sands Environmental Research Program Study Area
40.	WS 3.3	Mixing Characteristics of the Athabasca River below Fort McMurray - Winter Conditions
41.	AF 3.5.1	Acute and Chronic Toxicity of Vanadium to Fish
42.	TF 1.1.4	Analysis of Fur Production Records for Registered Traplines in the AOSERP Study Area, 1970-75
43.	TF 6.1	A Socioeconomic Evaluation of the Recreational Fish and Wildlife Resources in Alberta, with Particular Reference to the AOSERP Study Area. Volume I: Summary and Conclusions
44.	VE 3.1	Interim Report on Symptomology and Threshold Levels of Air Pollutant Injury to Vegetation, 1975 to 1978
45.	VE 3.3	Interim Report on Physiology and Mechanisms of Air-Borne Pollutant Injury to Vegetation, 1975 to 1978
46.	VE 3.4	Interim Report on Ecological Benchmarking and Biomonitoring for Detection of Air-Borne Pollutant Effects on Vegetation and Soils, 1975 to 1978.
47.	TF 1.1.1	A Visibility Bias Model for Aerial Surveys for Moose on the AOSERP Study Area
48.	HG 1.1	Interim Report on a Hydrogeological Investigation of the Muskeg River Basin, Alberta
49.	WS 1.3.3	The Ecology of Macrobenthic Invertebrate Communities in Hartley Creek, Northeastern Alberta
50.	ME 3.6	Literature Review on Pollution Deposition Processes
51.	HY 1.3	Interim Compilation of 1976 Suspended Sediment Date in the AOSERP Study Area
52.	ME 2.3.2	Plume Dispersion Measurements from an Oil Sands Extraction Plan, June 1977

53.	HY 3.1.2	Baseline States of Organic Constituents in the Athabasca River System Upstream of Fort McMurray
54.	WS 2.3	A Preliminary Study of Chemical and Microbial Characteristics of the Athabasca River in the Athabasca Oil Sands Area of Northeastern Alberta
55.	HY 2.6	Microbial Populations in the Athabasca River
56.	AF 3.2.1	The Acute Toxicity of Saline Groundwater and of Vanadium to Fish and Aquatic Invertebrates
57.	LS 2.3.1	Ecological Habitat Mapping of the AOSERP Study Area (Supplement): Phase I
58.	AF 2.0.2	Interim Report on Ecological Studies on the Lower Trophic Levels of Muskeg Rivers Within the Alberta Oil Sands Environmental Research Program Study Area
59.	TF 3.1	Semi-Aquatic Mammals: Annotated Bibliography
60.	WS 1.1.1	Synthesis of Surface Water Hydrology
61.	AF 4.5.2	An Intensive Study of the Fish Fauna of the Steepbank River Watershed of Northeastern Alberta
62.	TF 5.1	Amphibians and Reptiles in the AOSERP Study Area
63.		
64.	LS 21.6.1	A Review of the Baseline Data Relevant to the Impacts of Oil Sands Development on Large Mammals in the AOSERP Study Area
65.	LS 21.6.2	A Review of the Baseline Data Relevant to the Impacts of Oil Sands Development on Black Bears in the AOSERP Study Area
66.	AS 4.3.2	An Assessment of the Models LIRAQ and ADPIC for Application to the Athabasca Oil Sands Area
67.	WS 1.3.2	Aquatic Biological Investigations of the Muskeg River Watershed
68.	AS 1.5.3 AS 3.5.2	Air System Summer Field Study in the AOSERP Study Area, June 1977
69.	HS 40.1	Native Employment Patterns in Alberta's Athabasca Oil Sands Region
70.	LS 28.1.2	An Interim Report on the Insectivorous Animals in the AOSERP Study Area
71.	HY 2.2	Lake Acidification Potential in the Alberta Oil Sands Environmental Research Program Study Area
72.	LS 7.1.2	The Ecology of Five Major Species of Small Mammals in the AOSERP Study Area: A Review
73.	LS 23.2	Distribution, Abundance and Habitat Associations of Beavers, Muskrats, Mink and River Otters in the AOSERP Study Area, Northeastern Alberta
-	--	Interim Report to 1978
74.	AS 4.5	Air Quality Modelling and User Needs
75.	LS 2.1	Interim Report on the Soils Inventory of the AOSERP Study Area

76. AF 4.5.1 An Intensive Study of the Fish Fauna of the Muskeg River Watershed of Northeastern Alberta

77. HS 20.1 Overview of Local Economic Development in the Athabasca Oil Sands Region Since 1961.

78. LS 22.1.1 Habitat Relationships and Management of Terrestrial Birds in Northeastern Alberta.

These reports are not available upon request. For further information about availability and location of depositories, please contact:

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