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Degree: Master of Science

Title of Thesis:
ACUTE, SUBLETHAL EFFECTS OF 2-(THIOCYANOMETHYLTHIO) -
BENZOTHIAZOLE ON THE PHYSIOLOGY OF JUVENILE SALMONIDS

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ACUTE, SUBLETHAL, EFFECTS OF 2- (THIOCYANOMETHYLTHIO) - BENZOTHIAZOLE ON THE PHYSIOLOGY OF JUVENILE SALMONIDS

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14 August 1991 (date)
Abstract

2-(thiocyanomethylthio)-benzothiazole (TCMTB), is an antisapstain agent used in British Columbia’s lumber industry. Whilst it is known to be acutely lethal to fish at low concentrations, little is known of its sublethal effects. This study investigated the sublethal toxicity of TCMTB to juvenile salmonids (Oncorhynchus kisutch, O. tshawytscha and O. mykiss) with respect to the parameters of swimming performance, oxygen consumption, plasma glucose, plasma lactate, hematocrit, leucocrit and gill histopathology.

Assessment of swimming performance consisted of subjecting TCMTB pre-exposed fish to a series of increasing velocity increments within an annular swim chamber. Significant reductions in critical swimming speed occurred in a concentration-dependent manner, with thresholds of 5 - 10 μg·L⁻¹, depending on the species examined. The inhibitory effect of TCMTB on swimming performance was found to also be related to the duration of the exposure. Exposures of 12 - 24 h were the most critical with maximal reductions in performance occurring after 24 h exposure, regardless of concentration, over the range of 10.0 - 40.0 μg·L⁻¹.

Routine oxygen consumption rates were measured using an automated, intermittent-flow respirometer. Although oxygen consumption was influenced, its relationship to TCMTB concentration was not straightforward.

Plasma lactate, plasma glucose, hematocrit and leucocrit were assayed upon termination of the respirometer trials. Neither plasma glucose nor hematocrit were affected by a 48 h exposure to sublethal TCMTB
concentrations. Significant reductions in leucocrit and elevations in plasma lactate occurred in a concentration-dependent manner with thresholds of 15 and 7.5 µg·L⁻¹, respectively.

The respiratory exchange unit in fish gills is the secondary lamella. Lamellae from fish exposed to lethal and sublethal concentrations of TCMTB were examined histologically for structural aberrations. Quantitative observations of interlamellar distance and blood-water diffusion distance demonstrated significant alterations in lamellar morphology at concentrations as low as 6 µg·L⁻¹ TCMTB. Swelling of the sub-epithelial space significantly increased the blood-water diffusion distance and significantly decreased interlamellar distance.

Swimming speed, plasma lactate, leucocrit and gill histopathology, while varying in their convenience and applicability as sublethal indicators, were generally in good agreement with respect to the threshold concentrations (6 - 15 µg·L⁻¹) for sublethal TCMTB effects.

The observed alterations in critical swimming speed, plasma lactate and leucocrit indicate a physiological stress response to acute, sublethal TCMTB exposure. Reduced swimming performance is suggestive of impaired respiratory function. The presence of lamellar injury in response to TCMTB is consistent with the premise that disruption of gas-exchange capability by TCMTB may be a significant factor in this stress response.
Acknowledgements

The author wishes to thank Dr. A.P. Farrell for his support and encouragement throughout the project duration. I would also like to express my appreciation to the members of my supervisory committee; Dr. G. Kruzynski for his guidance, helpful suggestions and the use of his lab facilities, and Dr. R. Nicholson for his constructive criticism and interest during the preparation of this thesis. I am grateful also to Dr. B.A. McKeown for serving as public examiner of this thesis.

Many thanks are due to Jeff Johansen for his technical assistance. To the "donut-lab" crew at the Department of Fisheries and Oceans facilities, West Vancouver, B.C. and to Richard Stroub of the Environment Canada Chemistry Laboratory, West Vancouver, B.C. I would like to also express my appreciation. Chemicals, laboratory facilities and coho and chinook salmon stocks were generously provided by Dr. G. Kruzynski, DFO. Thanks also to Kwai-Yiu Lee for assistance with slide preparation.

This work was supported, in part, by an NSERC scholarship to the author, an NSERC operating grant to Dr. A.P. Farrell, and a PESTFUND grant to Dr. G. Kruzynski (DFO).

This study would not have been possible without the encouragement and support of my husband, Lee, and my family.
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Introduction:

British Columbia is the primary exporter of lumber products in Canada. In 1989 alone, approximately 40% of the world’s international softwood lumber trade originated in British Columbia (Agriculture Canada 1989). The coastal B.C. lumber industry is heavily dependent on the higher value offshore markets of Japan and the United Kingdom. The prolonged transportation and storage needed to reach these offshore markets increases the susceptibility of freshly cut softwood lumber to surface molds and fungal deterioration. The conditions found in shipping containers - mild temperatures, abundant moisture and minimal air circulation - favour the development of such fungi (Cserjesi and Johnson 1982). The fungi, which derive nourishment from food materials stored in the cells of the wood, cause an abnormal surface discoloration or “sapstain” on the lumber (Scheffer and Lindgren 1940). While most molds tend to cause only superficial discoloration, true sapstain fungi are able to penetrate the wood and cause deep and permanent stains. Failure to protect the wood from fungal discoloration can significantly reduce the value and marketability of the affected stock. Kiln drying of lumber can prevent sapstain discoloration by reducing the moisture content of the wood to a level which prevents proliferation of fungi. However, the high valued export markets are generally only for green wood products. Treatment of fresh sawn softwood lumber with fungicidal chemicals or “antisapstains” is thus considered necessary to meet the stringent lumber quality guidelines for export markets. Over 3.6 billion board feet of coastal softwood lumber, representing 91% of total production, were treated with antisapstain
chemicals in 1986, in response to these market demands (Agriculture Canada 1989).

Since the middle of the 1930’s sapstain preventive formulations used in North America have been based on chlorinated phenols (Scheffer and Lindgren 1940). However, due to concerns with regards to dioxin contamination, potential carcinogenicity, persistence in aquatic ecosystems and toxicity to aquatic organisms, the use of chlorophenate fungicides has become controversial in Canada (Agriculture Canada 1989). New regulations, repealing the registration of chlorophenates for sapstain control uses, have intensified the search for less toxic, alternative antisapstain chemicals. One alternative chemical currently registered and used by the B.C. forest industry is 2-(thiocyanomethylthio)-benzothiazole (TCMTB). TCMTB is the active ingredient in Busan 1030® and Busan 30WB®, which are antisapstain products produced by Buckman Laboratories (Memphis, Tennessee) for the wood protection industry. Like the chlorophenate fungicides, TCMTB provides a high degree of efficacy with regards to control of sapstain (Eslyn and Cassens 1983). However, as with the other wood preservative chemicals, concern exists over the toxicity of TCMTB; particularly the effects of TCMTB discharges into fishery waters. The aquatic environment can become exposed to these wood preservatives as a result of accidental spills, or more commonly, as a result of continuous low level leaching from treated lumber and lumber by-products. The economic, recreational and social importance of B.C.’s fisheries stocks must be considered and the environment supporting the fisheries fully protected. With an estimated wholesale value of approximately $584 million (1988),
B.C.’s salmon fishery, in particular, requires protection from deleterious chemicals (B.C. Ministry of Agriculture and Fisheries 1989).

With the goal of developing water quality criteria to adequately protect aquatic ecosystems, a number of laboratory-based bioassays have been formulated (Sprague 1971). These bioassays examine the response of an organism to concentrations of a toxicant. Until recently, acutely lethal bioassays, measuring mortality as the response factor, have provided most of the available toxicity data. The median lethal concentration (LC$_{50}$) of a toxicant that kills 50% of the test organisms within a specified time period, usually 96 hours (U.S. Environmental Protection Agency 1985), is used as a reference point for expressing the acute toxicity of a given toxicant. For TCMTB, 96-h LC$_{50}$ values of 17.3, 47.0 and 49.5 μg·L$^{-1}$ have been reported for coho salmon (Oncorhynchus kisutch), bluegill (Lepomis macrochirus) and rainbow trout (Oncorhynchus mykiss), respectively (G. Kruzynski, Toxicology Unit, West Vancouver Research Laboratory, Department of Fisheries and Oceans, West Vancouver, B.C., pers. comm.; Agriculture Canada 1989). By comparison, the 96-h LC$_{50}$ values for the chlorophenates indicate a range of toxicity two to three times less than that of TCMTB (salmonid 96-h LC$_{50}$’s range from 32.0 - 130.0 μg·L$^{-1}$)(Agriculture Canada 1989). Generally, an absence of information exists with regards to acute TCMTB toxicity in fish.

Lethality, however, is a very crude measure of acute toxic response. Sublethal effects occur at much lower concentrations of the toxic substance than those causing death, and so provide a more sensitive means of detecting its presence and potential environmental impact. There is an increasing awareness that determining and establishing safe limits for
effluent discharge will require measurements of various meaningful sublethal toxic responses of fish and other aquatic organisms.

Little published information exists about the sublethal effects of TCMTB. Increased "cough" frequency and disturbed gill integrity have been observed in salmonids exposed to sublethal concentrations of TCMTB (G. Kruzynski, pers. comm.). Juvenile coho salmon, *Oncorhynchus kisutch*, exposed to 10 μg·L⁻¹ TCMTB for 2 hours showed increases in coughing frequency to 17-20 coughs per minute as compared to 0-1 coughs per minute for control fish. The frequency of coughing (a brief reversal of water flow over the gills) was suggested as a quantifiable response to toxicants (Sprague 1971). Presumably, the cough reflex serves as a means of voiding the gills of foreign matter and eliminating clogging of the respiratory epithelium. Increased cough rates have been demonstrated for sublethal exposures of fish to bleached kraft pulpmill effluent (Davis 1973), copper (Drummond *et al.* 1973) and aluminum at low pH (Walker *et al.* 1988). Reduced efficiency of gas exchange and a concomitant reduction in oxygen utilization have also been associated with high cough frequency (Davis 1973).

Sublethal exposure to TCMTB has also been found to decrease juvenile coho survival at seawater entry (G. Kruzynski, pers. comm.). After a 1 week exposure to 5 μg·L⁻¹ of TCMTB in freshwater, no mortalities had occurred. However, after switching to saltwater, mortality reached 80% within 28 hours. Reduced seawater tolerance has been observed with sublethal concentrations of copper and zinc (Lewis and Lewis 1971) and DDT (Leadem *et al.* 1974). In these studies, it was believed that alterations in the gill ATPase enzyme system were responsible for the reductions observed.
In 1990, concentrations of TCMTB in the 200 to 400 µg·L⁻¹ range were recorded at outfall pipes emptying into the Fraser River from lumber treatment and storage facilities employing TCMTB as an antisapstain agent (G. Kruzynski, pers. comm.). On September 1, 1990, legislated maximum permissible levels of TCMTB in water discharged from lumber storage and treatment areas were reduced from 200 to 15 µg·L⁻¹ (B.C. Ministry of Environment 1989). However, even this new lower level of discharge may not provide adequate protection for aquatic organisms. Sublethal effects such as those mentioned above, occurred at TCMTB concentrations of 5 to 10 µg·L⁻¹, well below the 15 µg·L⁻¹ maximum limit. Given the paucity of toxicological information on TCMTB and the concentrations present in the aquatic environment, a thorough examination of acute and sublethal toxicity is necessary to fully determine the impact of TCMTB discharges on resident fish populations.

Many of the results obtained from sublethal toxicity experiments are thought to be based on a physiological stress response. Stress has been defined by Selye (1973) as: "the sum of all the physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of a physical or chemical force". Toxicant-induced stress responses may be produced either directly or indirectly by the particular agent and result in neural-hormonal imbalances. In fish, these endocrinological perturbations are believed to involve a pituitary-interrenal axis (adrenal cortical homologue) and in some respects resemble the stress reactions in mammals (Donaldson 1981). Additionally, stress may be reflected by changes in adrenergic function. While increased production and release of
corticosteroids follows activation of the pituitary-interrenal system, adrenergic stimulation inevitably results in an increase in the concentrations of plasma catecholamines (Mazeaud & Mazeaud 1981). Investigators have reported increases in corticosteroid and catecholamine concentrations in salmonids in response to the stress of exposure to copper (Donaldson and Dye 1975), suspended solids (Redding and Schreck 1987), acid/aluminum exposure (Goss and Wood 1988) and acute handling stress (Mazeaud et al. 1977, Barton et al. 1986). Secondary alterations in blood chemistry, hematology and metabolic function which reflect the effects of acute stressors have also been suggested as quantifiable parameters of stress (Wedemeyer and McLeay 1981). An understanding of both the physiology of the stress response and the ability of the fish to effect the required physiological adaptations is important when considering measurable indicators of sublethal toxicity.

The sublethal effect of toxicant stress on fish must also be examined in terms of its significance to the success of the fish in the environment. The ability to feed, avoid predation, maintain stream position and undergo spawning migration are all essential for survival and are directly dependent on fish swimming ability. Thus, Cairns (1966) suggested swimming performance as an important criterion in the determination of sublethal effects of toxicants on fish. Swimming performance has since been used to define tolerance limits to a number of pollutants and environmental parameters, including sodium pentachlorophenate (Webb and Brett 1973), bleached kraft pulpmill effluent (Howard 1975, McLeay and Brown 1979), pulpwood fibers (MacLeod and Smith 1966), cyanide (Kovacs and Leduc 1982), copper (Waiwood and Beamish 1978), pesticides (Peterson 1974,
Swimming performance in fish is usually separated into three categories: sustained, prolonged and burst speeds. Sustained speeds are supported primarily by aerobic metabolism and are maintained for long time periods (>200 min). Prolonged swim speeds are faster than sustained, but are maintained for less time (20 s - 200 min), and are dependent on both aerobic and anaerobic metabolism. Burst speeds, representing the maximum attainable speed, are mainly anaerobic and can only be maintained for seconds (Beamish 1978).

Critical swimming speed, a special category of prolonged swimming, is the measurement of choice as an indicator of sublethal toxicity (Sprague 1971). As defined by Brett (1964), the critical swim speed is the maximum velocity a fish can maintain for a specified time period. Under stepwise increases in velocity, the critical swim speed represents the final increment of velocity capable of causing fatigue and the simultaneous failure of all physiological systems (Jones 1971). Critical swimming speed is more rigorously defined by (Brett 1964):

\[ U_{crit} \text{ (critical swimming speed)} = u_i + \left( \frac{t_i}{t_{ii}} \times u_{ii} \right) \]

where \(u_i\) is the highest velocity maintained for the prescribed period (cm·s\(^{-1}\)); \(u_{ii}\) is the velocity increment (cm·s\(^{-1}\)); \(t_i\) is the time (min) the fish swam at the "fatigue" velocity; \(t_{ii}\) is the prescribed period of swimming (min). Reduced critical swimming speed in the presence of a pollutant
would appear to indicate an overall decline in swimming ability and presumably, survival capability.

Waiwood and Beamish (1978) described swimming performance as being sensitive to a number of toxic actions, including impairment of transport or exchange of respiratory gases, alterations in energy transformations, or inhibition of nervous or muscular activity. A suggested key mechanism by which some toxicants impair swimming performance is through reduction in active oxygen uptake (Sprague 1971, Satchell 1984). Those pollutants which act as general stressors without preferentially affecting respiratory gas exchange are not expected to alter swimming capabilities (Webb and Brett 1973). Although acute exposure to some toxicants may impair swimming performance, chronic exposures to the same toxicant may result in the compensatory development of a more efficient cardiorespiratory system which yields normal or improved swimming performance (Wedemeyer and McLeay 1981). Thus, the applicability of swim speed as a sublethal toxicity criterion is dependent on both the physiological mechanism of toxicant action and the nature of the toxicant exposure. Where ventilatory alterations are occurring, as has been observed with TCMTB exposure (G. Kruzynski, pers. comm.), changes in oxygen utilization and resultant affects on fish swimming performance would be expected.

Aerobic swimming performance and oxygen consumption rate are inseparably related to each other. Sprague (1971) has suggested oxygen consumption as an index of sublethal toxicity to fish and one which may have a direct limiting effect on aerobic performance. Both oxygen uptake by the gills and oxygen delivery to the tissues may be involved in this limiting
effect (Wood and Perry 1985). Waiwood and Beamish (1978) noted an increased oxygen consumption rate in trout swimming at controlled speeds following a sublethal copper exposure. Elevated resting or "routine" oxygen consumption rates have also been demonstrated in fish exposed to organochlorine insecticides (Waiwood and Johansen 1974) while organophosphorus insecticides have produced reductions in oxygen consumption (Rao et al. 1985). Exposure of juvenile coho salmon to the herbicide Garlon 4, produced variable results in routine oxygen consumption rates (Johansen and Geen 1990) while Janz et al. (1991), using various herbicides, detected no such alterations. Those toxicants affecting metabolic rate, and hence oxygen consumption rate, are believed to either limit oxygen consumption during maximum activity or increase oxygen consumption requirements associated with standard physiological maintenance (Fry 1971). Oxygen consumption rates thus serve as a general reflection of the energy expenditure of a fish undergoing a sublethal stress response.

Besides measurement of circulating levels of the primary stress hormones (catecholamines and corticosteroids), other blood chemistry and hematological characteristics of fish are useful and relatively simple indicators of sublethal response (Sprague 1971, Wedemeyer and McLeay 1981). Mobilization of muscle glycogen into blood sugar (reflecting increased metabolism, in general) and release of muscle lactate to the blood (reflecting increased anaerobic metabolism) are just two of the physiological consequences of increased levels of primary stress hormones (Mazeaud and Mazeaud 1981). As a result, both hyperglycemia and hyperlacticemia have been extensively utilized as measures of sublethal toxicant response
(Silbergeld 1974, McLeay 1977, Mazeaud et al. 1977, Wedemeyer and Yasutake 1977, Janz et al. 1991). Secondary changes in hematology that are proving to be useful indices include changes in the ratio of packed red cells (hematocrit) or packed white cells (leucocrit) to volume of whole blood (Sprague 1971, McLeay and Gordon 1977). Alterations in hematocrit and leucocrit have been utilized as indicators of sublethal pulp mill effluent and herbicide toxicity (McLeay and Gordon 1977, McLeay and Howard 1977, Janz et al. 1991). As with all clinical measures of toxicant-induced stress, our ability to interpret and understand the biological significance of changes in these parameters is crucial.

Histopathological gill lesions have also been reported as a response to both lethal and sublethal toxicant exposures (Skidmore and Tovell 1972, Hughes et al. 1979). Frequently recorded histopathological lesions include changes in gill epithelium (lifting, necrosis, hyperplasia, hypertrophy, rupture), fusion of gill lamellae, hypersecretion and proliferation of mucocytes, and changes in chloride cells and gill vasculature (Mallatt 1985). The nonspecificity of branchial alterations suggests that they primarily represent stereotyped physiological reactions of gills to stress rather than inflammatory responses (Mallatt 1985). Scott and Rogers (1980) suggested that the irritant-induced branchial responses may be mediated non-locally by the central regulatory systems of the fish. The development of characteristic gill lesions in fish exposed to stressors which do not directly encounter the gill (i.e. fish handling, i.p. injection) implies that at least some of these gill changes are controlled by the pituitary-interrenal axis, or by the central and autonomic nervous system (Mallatt 1985). The increased cough frequency in fish exposed to sublethal concentrations of TCMTB suggests some degree of gill
irritation or damage. Histological techniques may thus serve as a useful tool in examining this response.

The present study was undertaken to determine the effects of acute exposure of salmonids to sublethal concentrations of the antisapstain agent, TCMTB. A 96-h LC50 was first performed with chinook salmon to provide more extensive information on the acute lethality of TCMTB and to provide concentration ranges for subsequent sublethal studies. Sublethal effects were gauged using swimming performance, oxygen consumption, plasma glucose and lactate, hematocrit, leucocrit and gill histopathology in TCMTB-stressed juvenile coho salmon, Oncorhynchus kisutch, chinook salmon, O. tshawytscha, and rainbow trout, O. mykiss. Experiments were undertaken with the purpose of predicting the impacts of sublethal TCMTB exposure on salmonid biochemical and physiological condition. A comparative examination of these various parameters was also undertaken to assess their applicability as indicators of sublethal toxicity in fish.
Materials and Methods:

Test Animals and Test Chemical:

Juvenile chinook salmon (*Oncorhynchus tshawytscha*) used as the test species for the acute, lethal bioassays and the initial swimming speed trials, were Harrison River stock obtained from Chilliwack Fish Hatchery, Chilliwack, B.C. in early spring 1989. The bioassay fish (ca. 3-10g) were maintained in outdoor 3800-L fiberglass tanks at the Department of Fisheries and Oceans (DFO) facilities, West Vancouver. The tanks were serviced by flow-through well water (pH 6.8-6.9, O$_2$ saturation >95%) and fish stocks were held outside, under a natural spring photoperiod. Some of this stock of fish were transferred to Simon Fraser University (SFU) on June 27, 1989, where swimming trials were conducted. Yearling rainbow trout (*Oncorhynchus mykiss*) (ca. 20-30g) and coho salmon (*Oncorhynchus kisutch*) juveniles (ca. 10-20g), to be used for the remainder of the experiments, were obtained from the West Creek Trout Farm, Abbotsford, B.C., on August 11, 1989 and the Capilano Hatchery on August 28, 1989, respectively. Fish were held at SFU under natural photoperiod, in 2000-L fiberglass tanks supplied with dechlorinated municipal water of pH 6.1-6.7, O$_2$ saturation >95% and hardness 5.2-6.0 mg·L$^{-1}$ CaCO$_3$. Fish were fed daily to satiation on a commercial pellet diet (Oregon Moist); pellet size was adjusted as fish increased in size.

In its pure form, 2-(thiocyanomethylthio)-benzothiazole (TCMTB) is a white, crystalline material of low aqueous solubility (Cserjesi and Johnson 1982)
The physical and chemical properties of TCMTB are outlined further in Table 1. Technical grade TCMTB, a black viscous liquid, forms the active ingredient of the commercial fungicide Woodstat 30WB®, comprising 30% of the product. A small percentage of technical grade TCMTB consists of impurities of unknown chemical composition. The remainder of the commercial product consists of a proprietary, water-soluble carrier. Both Woodstat 30WB® and the carrier are products of Buckman Laboratories (Memphis, Tennessee). In the present study, the commercial product was used as the source of TCMTB, with reported concentrations based on the technical grade TCMTB content of the commercial formulation. Biological effects measured thus represent the combined effect of pure TCMTB and its technical impurities.

Stock solutions of TCMTB and the carrier solution were prepared separately for each experimental trial using glass-distilled, deionized water. To prevent degradation of active ingredient, TCMTB stocks were stored in opaque, glass flasks and replaced, at a minimum, every four days.

Acute, Lethal Bioassays:

Two separate bioassays were conducted at the DFO laboratory facilities in West Vancouver over the period of June 5-16, 1989. For the first bioassay, three TCMTB concentrations were tested, based on a logarithmic progression bracketing 96-h LC₅₀ values obtained for other salmonid species (G. Kruzynski, pers. comm., Ward 1989). The second bioassay used three test
FIG. 1. Chemical structure of 2-(thiocyanomethylthio)-benzothiazole (TCMTB).
TABLE 1. Physical and chemical properties of TCMTB.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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| Water solubility                | 40 mg L\(^{-1}\) at 24\(^\circ\)C  
16 mg L\(^{-1}\) at 5\(^\circ\)C   |
| Vapour pressure                 | 3.6 x 10\(^{-6}\) torr                     |
| Boiling point                   | >120\(^\circ\)C                           |
| Specific gravity                | 1.38                                       |
| \(K_{ow}\) value                | 25 (log \(K_{ow}\) = 1.40)               |
| Persistence in soil             | \(T_{1/2} = 23\) h                      |
| Persistence in water            | \(T_{1/2} = 15\) days  
(at pH 7.0 and under aerobic conditions) |

(Agriculture Canada 1989; Ward 1989)
concentrations which bracketed the value obtained in the first bioassay. Both bioassays included, as a control exposure, a concentration of carrier solution equivalent to the highest test concentration employed (i.e. 40.0 \(\mu g\cdot L^{-1}\) for the first and 20.0 \(\mu g\cdot L^{-1}\) for the second bioassay). Lethality testing was conducted in accordance with standard procedures outlined by U.S. EPA (1985) for continuous-flow, acute bioassays.

Sixty chinook fry (mean weight 5.3 g, SEM=1.2; mean fork length 7.9 cm, SEM=0.5) were randomly selected and equally distributed among four 70-L annular fiberglass chambers (containing 58 L of water) and acclimated for 48 hours prior to initiation of each bioassay. Holding tank temperatures of 11.0 and 11.5 °C and test chamber temperatures of 12.2 and 14.1 °C were recorded at the time of fish transfer, for the first and second bioassays, respectively. During transfer from holding tanks to test chambers the fish were anesthetized using an aerated solution of 0.12 g MS222 (Syndel) in 6 L of 2 parts freshwater to 1 part seawater. Fish were not fed 24 hours prior to transfer nor during acclimation and test periods. Flow-through dilution water used in the bioassays was identical to that used for maintenance and acclimation purposes. Centrifugal pumps (March MDX) circulated water through the test chambers, generating an average current velocity of 10.6 \(\pm\) 0.6 cm\(\cdot\)s\(^{-1}\). Midstream velocities were measured in the chambers prior to each bioassay and adjusted accordingly. Constant water inflow was maintained at 1000 mL\(\cdot\)min\(^{-1}\) using Manostat predictability flowmeters. Waste traps and a standpipe drainage system served to remove debris and maintain water levels within the chambers.
The toxicant delivery system consisted of a head chamber (18 L Mariotte bottle) containing the particular TCMTB dilution being tested and a Teflon tubing dosing apparatus. A pressure differential between the inflow tube at the head chamber and the outflow tube at the bioassay chamber was used to regulate the toxicant drip rate. Test solution delivery was maintained at a constant rate over the 96 hour period by production of a vacuum within the head chamber. Drip rates were monitored throughout the bioassays.

Testing was conducted at water temperatures of $13.4 \pm 1.0^\circ C$ and $12.6 \pm 0.7^\circ C$ for the first and second bioassays, respectively. Water temperature was automatically recorded hourly at both the header tank and test chambers. A 14 h light; 8 h dark; 1 h dawn and dusk photoperiod was maintained throughout the bioassays. Dissolved oxygen and pH were monitored on a daily basis (Leeds and Northrup digital oxygen meter, Radiometer Copenhagen pH meter). Test chambers were frequently monitored for mortality throughout the 96 hour period.

Swimming Performance Experiments:

Three sets of experiments were conducted to evaluate the effect of sublethal TCMTB concentrations on swimming performance. The initial swim trials were performed with chinook juveniles (mean weight 27.5 g, SEM=1.6; fork length 14.1 cm, SEM=0.2) during July-August 1989. A second set of trials, examining the effects of varying both TCMTB exposure time and concentration, was performed with rainbow trout (mean weight 27.1 g, SEM=0.5; fork length 12.8 cm, SEM=0.1) during August-September 1989. The final study, performed in July-August 1990, further examined the effects
of varying exposure time and concentration but used coho juveniles (mean weight 24.7 g, SEM = 0.6; fork length 13.8 cm, SEM = 0.1) as the test animal. Sublethal TCMTB test concentrations evaluated were based on the previously obtained bioassay results for chinook salmon, and on 96-h LC50 values of 49.5 \( \mu g L^{-1} \) (static bioassay) and 17.3 \( \mu g L^{-1} \) (continuous-flow) for rainbow trout and coho salmon, respectively (Ward 1989, G. Kruzynski, pers. comm.). Both chinook and coho were exposed to Woodstat 30WB\( ^{\circ} \) giving nominal TCMTB concentrations of 5.0, 7.5, 10.0, 15.0 and 20.0 \( \mu g L^{-1} \), whereas rainbow trout were exposed to Woodstat 30WB\( ^{\circ} \) giving nominal TCMTB concentrations of 5.0, 10.0, 20.0 and 40.0 \( \mu g L^{-1} \). A 0 \( \mu g L^{-1} \) control and a carrier solution equivalent to the highest TCMTB concentration (20.0 \( \mu g L^{-1} \) for chinook and coho, 40.0 \( \mu g L^{-1} \) for rainbow trout) were also employed during the exposure period.

Fish used in the first and second trials were pre-exposed to the TCMTB solution in 18-L plastic buckets equipped with external standpipes and activated charcoal effluent filters. The TCMTB dilution apparatus employed a Masterflex variable speed peristaltic pump to deliver a known concentration of TCMTB from an 18-L reservoir to the tanks' incoming water supply at a constant rate (4.53 mL-min\(^{-1}\)). The same peristaltic pump also controlled the rate of water flow (250 mL-min\(^{-1}\)) from the header tank to the exposure tanks, ensuring 90% replacement of water in approximately 3 hours. All tanks, experimental and header, were equipped with airstones to drive off excess \( CO_2 \) and saturate with \( O_2 \). Dissolved oxygen was monitored (YSI portable \( O_2 \) meter) throughout the exposure to ensure \( O_2 \) saturation >95%. Water temperature and pH were monitored daily. Chinook and rainbow
trout TCMTB exposures were conducted at temperatures of 13.9 ± 0.1 °C and 14.2 ± 0.2 °C, respectively.

Chinook (5 fish per concentration) were pre-exposed to the TCMTB solutions for 48 hours prior to the initiation of swim trials. In addition to the regular 48 hour TCMTB exposure, rainbow trout within the 40 µg·L⁻¹ treatment group also received 12, 16 and 24 hour exposures. Five trout were utilized at each concentration and all trials were performed in duplicate. Fish loading densities during the exposure phase were approximately 0.4 g fish·L⁻¹·day⁻¹. Immediately following the exposure periods the fish were transferred to the swimming chamber and allowed an acclimation period (12 h for chinook, 4 h for rainbow trout) prior to the actual test of swimming ability. Initial water temperatures in the swimming chambers were 13.5 ± 0.7 °C and 13.4 ± 0.6 °C, for the chinook and rainbow trout trials, respectively.

Juvenile coho salmon used in the final swim speed trials (5 fish per trial) were pre-exposed to their respective TCMTB concentrations in 8-L glass respirometer vessels, at an approximate loading density of 0.8 g fish·L⁻¹·day⁻¹. A computer-controlled delivery pump and valve system ensured that appropriate TCMTB concentrations were maintained. Dissolved oxygen and water temperature were also automatically recorded, with mean water temperatures during this series of TCMTB exposures at 14.7 ± 0.1 °C. The respirometer is described in more detail in the section on "oxygen consumption experiments". Each toxicant exposure was preceded by a 16-24 h continuous-flow acclimation to the respirometer vessels. Following the
exposure period, fish were transferred to the swimming chamber and allowed a 4 h acclimation at rest prior to the actual test of swimming ability. Water temperatures in the swim chamber at the time of transfer were $15.1 \pm 0.5 \, ^\circ C$. Two replicate trials were conducted for each TCMTB concentration, including the carrier solution, using a 48 h exposure period. A single trial, again with 5 fish per test, was performed for each TCMTB concentration after 12, 18 and 24 h exposure periods.

The swim testing apparatus consisted of a 2470-L ovoid, fiberglass raceway tank (outside length 343 cm, depth 41 cm, raceway width 46 cm) outfitted with two variable output propulsion motors (Figure 2). Two enclosed, cylindrical testing chambers were located within each straight section of the tank. Turning vanes, screens and contraction cones, located upstream of each test chamber, corrected rotational disturbances introduced by the propellers and smoothed the velocity profile within the test sections. Water velocity, controlled by regulating voltage output to the propulsion motors, was pre-calibrated with a portable current meter.

Critical swimming speed was expressed as bodylengths per second (BL·s$^{-1}$) following the procedures developed by Brett (1964). For coho trials initial velocity was 0.20 m·s$^{-1}$ and the speed was increased in increments of 0.05 m·s$^{-1}$ at 15-min intervals up to the fatigue velocity. Increments of 0.10 m·s$^{-1}$ at 30-min intervals were employed for the chinook and trout trials.
FIG. 2. Schematic drawing of apparatus employed for critical swim speed testing.
Fatigued fish were removed from the test chambers individually via a movable screen gate, at which time fork length and weight were recorded.

**Oxygen Consumption Experiments:**

Oxygen consumption trials were performed with juvenile coho salmon (mean weight 24.7 g, SEM=0.6; fork length 13.8 cm, SEM=0.1) from 27 March to 29 June 1990 in an eight vessel, computer-controlled, intermittent-flow respirometer similar to that of Duval *et al.* (1981), with modifications as per Johansen and Geen (1990) (Figure 3). This respirometer design allows freshwater flushing and oxygen consumption measurements within each vessel every 30 minutes. Appropriate TCMTB concentrations were maintained in the vessels by computer control of delivery pump timing. The software design allowed user control of diluent water flow and toxicant delivery via a series of solenoid valves. Control of duration of pre-exposure, exposure and recovery periods and temperature monitoring ability was also provided. External disturbances were minimized by blacking-out the vessels with opaque polyethylene covers. Water temperatures throughout the trials ranged from 10.5 to 13.0°C and a 14 h light:10 h dark (30 min for dawn and dusk) photoperiod was maintained.

Fish were weighed, placed in the 8-L vessels (5 per vessel) and allowed to acclimate under continuous-flow conditions of ca. 1 L·min⁻¹ for a 24-h period. Food was withheld 24 h before transfer to the vessels as well as
FIG. 3. Diagrammatic drawing of the respirometer apparatus used for measurement of routine oxygen consumption.
during all experimental trials. Trials consisted of a 48 h toxicant pre-exposure period followed by a 48 h exposure period, starting and ending at 1300 hours. Fish were exposed to Woodstat 30W® giving nominal TCMTB concentrations of 5.0, 7.5, 10.0, 15.0 and 20.0 µg·L⁻¹, respectively. A 0 µg·L⁻¹ control and a 20.0 µg·L⁻¹ equivalent carrier solution were also employed during the exposure period. Three vessels without fish received 0 and 20.0 µg·L⁻¹ TCMTB and a 20.0 µg·L⁻¹ carrier solution, respectively, to correct for chemical oxygen demand, if any. Five replicate trials were conducted for each TCMTB concentration as well as a single carrier trial.

Oxygen consumption rates were based on the pooled values for 5 fish within a single respirometer vessel, as measured every 30 minutes. This mass-specific rate is based on the total wet weight of the five fish in each of the test vessels. These values were averaged over 24-h periods throughout the trial and means of replicate trials were taken to obtain the final values. The effect of TCMTB on oxygen consumption was tested in two ways: (1) comparison of each TCMTB exposure value with its corresponding control exposure value, and (2) comparison of the differences between pre-exposure (24-48 h) and exposure (0-24 and 24-48 h) values for each TCMTB concentration. Since fish exhibit diurnal rhythms of oxygen consumption (Brett and Zala 1975), it was preferable to compare mean values for the full 24 h cycle rather than specific values within the cycle. A characteristically elevated oxygen consumption during the initial 0-24 h pre-exposure period precluded the use of this period for statistical analysis (see Figure 4).
FIG. 4. Daily oxygen consumption patterns of coho salmon (*Oncorhynchus kisutch*) exposed to 20.0 μg·L⁻¹ TCMTB in comparison to control fish (N=25).

Note: Dark bars on time axis represent a lights-off situation.
Hematological Analyses:

Blood was sampled from the juvenile coho upon termination of the respirometer trials. This blood was used to assay for plasma lactate and plasma glucose concentrations, hematocrit and leucocrit. To minimize handling stress, the sampling was conducted as follows (Janz et al. 1991): Immediately following the final respirometer exposure cycle, 24 mL of 50% 2-phenoxyethanol in distilled water was injected into each vessel. Anesthetized fish were then sampled sequentially, with a total sample collection time of ca. 3 minutes per vessel. Blood was collected from the severed caudal peduncle into individual 75 µL microhematocrit tubes pretreated with ammonium-heparin. After centrifugation in an IEC clinical centrifuge (Model PR-6) (5 min at 2,000 RPM) hematocrit was determined as percent packed red cell volume. Leucocrit (volume of packed leucocytes and thrombocytes as a percentage of whole blood volume) was determined according to the procedure described by McLeay and Gordon (1977). Hematocrit and leucocrit were determined for each fish. Individual values were pooled for each treatment group.

Blood plasma was retrieved by breaking the microhematocrit tubes above the packed leucocytes. All plasma showing evidence of hemolysis was discarded. Plasma was then pooled for each treatment group, deproteinated with 8% HClO₃ and stored at 4 °C until subsequent analysis. Plasma lactate levels were determined enzymatically using the L-lactate dehydrogenase/NADH method described by Loomis (1967). Plasma glucose
levels were determined by the enzymatic (hexokinase) method of Bondar and Mead (1974). Plasma lactate and glucose determinations were performed at 340 nm using a Spectronic 21 spectrophotometer (Bausch and Lomb) and Sigma pre-mixed reagents. Duplicate assays were performed on plasma samples obtained from two of the five replicate trials.

**Gill Histology:**

Gills were examined histologically to relate any structural aberrations to exposure interval and concentration. Secondary lamellae of juvenile chinook from the bioassays of June 5-16, 1989 were analyzed for structural changes using morphometric methods. Fish were removed from the bioassay chambers either as they became moribund or at the termination of the bioassays. Exact details on the number of fish sampled at each concentration and the duration of the exposures received are presented later (see Table 5). Fork length and weight were recorded and specimens were immediately fixed in phosphate-buffered 10% formalin, pH 7.2, (prepared as per Vacca 1985). Formalin solutions were changed four times within the 48 h period following the initial fixation. Gill arches were then dissected free and two 1 mm sections were cut away from the second left gill arch of each of the fish collected and immediately washed in distilled water. The samples were then dehydrated in increasing concentrations of ethanol (30% for 48 h, 50% for 24 h, and 70% for 24 h), cleared in xylene and embedded in paraffin wax. Oriented 5-8 μm sections were mounted on glass slides and stained with hematoxylin and eosin. The sections were examined with a light microscope fitted with a Prado Universal microscopic
projector using a 40X objective. An Apple IIC microcomputer equipped with a digitizing graphics tablet and graphics tablet software was used to measure distances on the projected section images. Screen distances were pre-calibrated using a stage micrometer projected onto the digitizing tablet. Five fields were examined for each prepared section, with 10 gill measurements performed within each field. All measurements were made midway between the distal and proximal ends of the secondary lamellae (see Figure 5). Interlamellar distance was defined as the distance between the outer epithelial surfaces of two adjacent secondary lamellae. Blood-water diffusion distance was taken as the distance between the outer epithelial surface and the nearest blood space on any given lamellae.

**Quality Assurance:**

In the two acute, lethal bioassays, water samples taken after 72 h at each concentration level were used to ascertain the actual exposure concentration. Sampling at each concentration on the final day of exposure was also conducted during both the coho swimming performance and oxygen consumption trials. All samples were immediately covered to exclude light and refrigerated (4°C) to minimize deterioration of the active ingredient. High-performance liquid chromatography (HPLC) analysis for 2-(thiocyanomethylthio)-benzothiazole and its degradation products was performed by the Environment Canada Chemistry Laboratory, West Vancouver, B.C.
FIG. 5. Simplified diagram of two secondary lamellae in transverse section, illustrating the parameters used for the gill histopathology studies. Abbreviations: EP, epithelium; BLS, blood spaces; PC, pillar cell; SL, secondary lamellae; ILD, interlamellar distance; BWDD, blood-water diffusion distance.
The resulting quality control analyses for active TCMTB ingredient are presented in Table 2. Concentrations of TCMTB were below the HPLC detection limit of 5.0 μg·L⁻¹ in all control samples. The allowable precision error around the detection value is taken as a standard deviation of ± 7.4 μg·L⁻¹ over 5 consecutive samples (Richard Stroub, Environment Canada Chemistry Laboratory, West Vancouver, B.C., pers. comm.), representing an acceptable standard error of 3.3 μg·L⁻¹. All samples analyzed fell within allowable detection ranges, with the exception of a single 15.0 μg·L⁻¹ sample, analyzed as 22 μg·L⁻¹. As the number of measured determinations of TCMTB concentrations was limited, the test results are reported as nominal concentrations, barring the two acute toxicity bioassays, where both nominal and measured values are reported.

Data Analyses:

Median acute lethal values (96-h LC₅₀'s) and their 95% confidence limits were calculated by computer using binomial probability analysis as per Stephan (1977). These values were confirmed by graphical log-probit analysis, and where possible by computerized probit and moving average analysis. Values for measured rather than nominal concentrations of TCMTB were also calculated. Confidence limits (95%) were, where data permitted, calculated along with the 96-h LC₅₀ value.

Differences between the mean critical swimming speeds, oxygen
TABLE 2. Quality assurance analysis: Nominal versus measured concentrations of TCMTB detected in water samples as analyzed by HPLC.

<table>
<thead>
<tr>
<th>TCMTB Concentrations (µg·L⁻¹)</th>
<th>Sample 1ᵃ</th>
<th>Sample 2ᵇ</th>
<th>Sample 3ᶜ</th>
<th>Sample 4ᵈ</th>
<th>MEAN MEAS. (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOM. MEAS. *</td>
<td>NOM. MEAS.</td>
<td>NOM. MEAS.</td>
<td>NOM. MEAS.</td>
<td>NOM. MEAS.</td>
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</tr>
<tr>
<td>20.0 10.0</td>
<td>20.0 14.0</td>
<td>20.0 18.0</td>
<td>20.0 22.0</td>
<td>16.0</td>
<td>(2.6)</td>
</tr>
<tr>
<td>- -</td>
<td>- -</td>
<td>15.0 15.0</td>
<td>15.0 22.0</td>
<td>18.5</td>
<td>(3.5)</td>
</tr>
<tr>
<td>10.0 7.0</td>
<td>10.0 11.0</td>
<td>10.0 10.0</td>
<td>10.0 19.0</td>
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<td>(2.6)</td>
</tr>
<tr>
<td>- -</td>
<td>- -</td>
<td>7.5 7.0</td>
<td>7.5 12.0</td>
<td>9.5</td>
<td>(2.5)</td>
</tr>
<tr>
<td>- -</td>
<td>6.0 6.0</td>
<td>- -</td>
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<td>- -</td>
<td>- -</td>
<td>5.0 6.0</td>
<td>5.0 12.0</td>
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<td>(3.0)</td>
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<td>0 &lt;5</td>
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<td>&lt;5</td>
</tr>
</tbody>
</table>

ᵃ Sample 1: First acute, lethal bioassay @ 72 h exposure
ᵇ Sample 2: Second acute, lethal bioassay @ 72 h exposure
ᶜ Sample 3: O₂ consumption experiment @ approx. 48 h exposure
ᵈ Sample 4: Swim speed experiment @ approx. 48 h exposure
ᵉ HPLC detection limit = 5.0 µg·L⁻¹ TCMTB

* NOM. and MEAS. refer to “Nominal” and “Measured” concentrations of TCMTB, respectively
consumption, and blood parameters of the control, carrier and treatment groups were compared using an unpaired Student's t-test. A paired Student's t-test was used for statistical comparison between pre-exposure and post-exposure oxygen consumption rates within the same group of fish. One-way analysis of variance (ANOVA) and the Student-Neuman-Keuls test for comparisons of means were applied, as appropriate, to data obtained from the swimming experiments using variable exposure times. ANOVA was also used for comparisons between gill morphological parameters at different TCMTB exposure concentrations. Significance was accepted at the 95% level (P<0.05).
Results:

Acute, Lethal Bioassays:

96-h LC$_{50}$ values of 10.3 µg·L$^{-1}$ and 11.4 µg·L$^{-1}$, for the first and second chinook salmon bioassays, respectively, were derived by binomial probability analysis (Table 3). It was not possible to calculate 96-h LC$_{50}$ values for the first bioassay using the moving average or probit methods. For the second bioassay, the moving average method and probit method produced 96-h LC$_{50}$ values of 10.9 (9.2, 12.9) and 10.7 (9.0, 13.2), respectively (bracketed values represent 95% confidence limits). Graphical estimation of the 96-h LC$_{50}$ value is presented in Figure 6.

Using binomial probability analysis, the 96-h LC$_{50}$ values for the actual measured concentrations were determined to be 7.3 and 11.5 µg·L$^{-1}$, for the first and second bioassays, respectively. Once again, it was not possible to generate an LC$_{50}$ value for the first bioassay using either the moving average or probit methods. Similarly, the probit method was not recommended (Stephan 1977) for use with the second bioassay. Moving average analysis of the measured concentrations in the second bioassay generated an LC$_{50}$ value of 10.3 µg L$^{-1}$ (9.2, 11.6).
TABLE 3. Continuous-flow, acute (96 h), lethal bioassay results for TCMTB-exposed juvenile chinook salmon (*Oncorhynchus tshawytscha*).**

<table>
<thead>
<tr>
<th>Bioassay #1</th>
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<td></td>
<td>percent survival</td>
<td>96h LC₅₀* (μg/L⁻¹)</td>
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<tr>
<td>test conc. (μg/L⁻¹)</td>
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<td>f</td>
<td>i</td>
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<td>pH</td>
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<td>96h LC₅₀* (μg/L⁻¹)</td>
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<tr>
<td>test conc. (μg/L⁻¹)</td>
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<td>7.0</td>
<td>10.1</td>
<td>10.3</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

i = initial value  
f = final value  
* 96-h LC₅₀ value derived by binomial probability analysis  
** N = 15 per concentration  
Note: Water temperature throughout the two bioassays averaged 12.6 ± 0.7 °C.
FIG. 6. Graphical estimation of the 96-h LC$_{50}$ for chinook salmon
(*Oncorhynchus tshawytscha*) exposed to TCMTB. Mortality data from both
the first bioassay (05/06/89) and the second bioassay (12/06/89) has been
included.
Swimming Performance Experiments:

The mean critical swimming speed of control chinook was 5.48 BL·s⁻¹ (SEM=0.23), reflecting a sustained velocity of 0.76 m·s⁻¹. After the 48 h exposure, critical swimming speeds decreased significantly with increasing TCMTB concentrations (Figure 7), starting at 5.0 µg·L⁻¹ (ca 45% of the 96-h LC₅₀ value). Swim speed was reduced by 11, 17, 16, 23 and 42% for 5.0, 7.5, 10.0, 15.0 and 20.0 µg·L⁻¹ TCMTB, respectively. Critical swimming speed was not affected after exposure to the carrier solution (5.51±0.16 BL·s⁻¹).

For rainbow trout controls, mean critical swimming speed was 5.27 BL·s⁻¹ (SEM=0.26), representing an actual sustained velocity of 0.69 m·s⁻¹. As with chinook salmon juveniles, critical swimming speeds were shown to decrease significantly with increasing TCMTB exposure concentration (Figure 8), beginning at a concentration of 10.0 µg·L⁻¹ (ca 20% of the 96-h LC₅₀ value). Swim speed was reduced by 11, 23 and 44% at concentrations of 10.0, 20.0 and 40.0 µg·L⁻¹ TCMTB, respectively. No effect on critical swimming speed was apparent after exposure to the carrier solution (5.25 BL·s⁻¹, SEM=0.10). In the 40.0 µg·L⁻¹ group a single mortality occurred during each of the 24 and 48 h exposure periods prior to the initiation of swim testing.

For a given concentration (40.0 µg·L⁻¹), critical swimming speed also decreased with increasing exposure time (Figure 9). Statistically significant
FIG. 7. Mean critical swimming speed in relation to TCMTB exposure concentration (48 h) for juvenile chinook salmon (*Oncorhynchus tshawytscha*) (*N*= 5) of mean fork length (BL) = 14.1 cm (SEM = 0.2). The 96-h LC50 value for chinook salmon is also shown. Vertical bars indicate SEM.

* Significant difference from control (*P* < 0.05)
FIG. 8. Mean critical swimming speed in relation to TCMTB exposure concentration (48 h) for juvenile rainbow trout (Oncorhynchus mykiss) (N=10) of mean fork length (BL) = 12.8 cm (SEM=0.1). The 96-h LC50 value for rainbow trout is also shown. Vertical bars indicate SEM. * Significant difference from control (P<0.05)
Swim Speed (L/s)

Rainbow Trout 96-h LC₅₀

TCMTB Conc. (ppb)
FIG. 9. Mean critical swimming speed of juvenile rainbow trout
(Oncorhynchus mykiss) (mean length, BL, = 12.8 cm, SEM=0.1) in relation
to duration of exposure to 40.0 μg·L⁻¹ TCMTB. N=10 for all control fish;
N=9 for 48 h exposure; N=5 for 12, 16 and 24 h exposures. Vertical bars
indicate SEM.
* Significant difference from control (P<0.05)
Swim Speed (L/s)

Exposure Period (h)
reductions were observed after 16 and 24 h exposures, as well as at the standard 48 h exposure. An apparent, though not significant reduction also occurred after a 12 h exposure. Mean swimming speed declined most rapidly for fish receiving exposure times between 12 and 24 h. Swimming performance did not appear to decline thereafter since the 48 h exposure results were similar to the 24 h exposure results.

The mean critical swimming speed of control fish for the coho test group was 6.29 BL·s⁻¹ (SEM=0.07), reflecting a sustained velocity of 0.86 m·s⁻¹. After the 48 h exposure, critical swimming speeds decreased significantly with increasing TCMTB concentrations (Figure 10), starting at 10.0 µg·L⁻¹ (ca 60% of the 96-h LC₅₀ value). Swim speed was reduced by 11, 19 and 25% for 10.0, 15.0 and 20.0 µg·L⁻¹ TCMTB, respectively. No effect on critical swimming speed was apparent after exposure to the carrier solution (6.26 BL·s⁻¹, SEM=0.07). In the 20.0 µg·L⁻¹ group, three mortalities occurred during the first hour of recovery from swimming to fatigue.

For all concentrations greater than or equal to 10.0 µg·L⁻¹, critical swimming speed decreased with increasing exposure time (Figure 11). Significant reductions in performance were observed after an 18 h exposure period for both the 15.0 and 20.0 µg·L⁻¹ treatment groups and after a 24 h exposure for the 10.0, 15.0 and 20.0 µg·L⁻¹ groups. Mean swimming speed declined most rapidly after an exposure period of between 12 to 18 h for the 15.0 and 20.0 µg·L⁻¹ group and between 18 to 24 h for the 10.0 µg·L⁻¹ group. TCMTB
FIG. 10. Mean critical swimming speed in relation to TCMTB exposure concentration (48 h) for juvenile coho salmon (*Oncorhynchus kisutch*) (*N=10*) of mean fork length (BL) = 13.8 cm (SEM=0.1). The 96-h LC$_{50}$ for coho salmon is also shown. Vertical bars indicate SEM.

* Significant difference from control (P<0.05)
FIG. 11. Mean critical swimming speed of juvenile coho salmon (Oncorhynchus kisutch) (mean length, BL, = 13.8 cm, SEM=0.1) in relation to duration and concentration of TCMTB exposure. N=10 for 48 h exposures; N=5 for all other exposure durations.
* Significant difference from control (P<0.05)
concentrations less than 10.0 μg·L⁻¹ did not affect critical swimming speed, regardless of the length of the exposure period employed here (up to 48 h).

Of interest, the rainbow trout swimming speed decreased by approximately half at concentrations lower than the 96-h LC₅₀ value for this species. By comparison, a similar decrease in swimming speed for chinook was not evident until toxicant concentrations well in excess of the 96-h LC₅₀ value were reached. The range of concentrations tested for coho salmon included only a single concentration (20.0 μg·L⁻¹) that was greater than their 96-h LC₅₀ value. At this concentration the extent of reduction in swimming speed was similar to that observed for chinook salmon and well below the decrease observed for rainbow trout.

**Oxygen Consumption Experiments:**

The mean oxygen consumption rate of 91 mg O₂·kg⁻¹·h⁻¹ (SEM=1.7) for the carrier solution was not significantly different from the control, though a slight but not significant elevation to 102 mg O₂·kg⁻¹·h⁻¹ (SEM=2.9) did occur during the 0-24 h post-exposure interval. Neither TCMTB nor the carrier compound caused significant oxygen uptake at 20.0 μg·L⁻¹ in the vessels when no fish were present (Figure 12). One mortality occurred within the 20.0 μg·L⁻¹ TCMTB group at 46 hours into the exposure. There was no significant change in the control oxygen consumption rate during the final three 24 h periods of the experiment.
FIG. 12. Chemical and biochemical oxygen consumption in respirometer vessels in the absence of fish.
A simple relationship between concentration of TCMTB and oxygen consumption was not apparent. In all treatment groups, the first exposure period (0-24 h) had a lower oxygen consumption rate than its paired pre-exposure value, though this effect was statistically significant only at 5.0 and 20.0 µg·L⁻¹ (Table 4). During the second exposure period (24-48 h), oxygen consumption either remained at this same low level (7.5 and 20.0 µg·L⁻¹), or showed a further decrease (15.0 µg·L⁻¹), or increased to or above the pre-exposure level (5.0 and 10.0 µg·L⁻¹). Thus, at lower TCMTB concentrations (5.0 - 15.0 µg·L⁻¹) recovery was possible after an initially reduced oxygen consumption rate. At TCMTB concentrations ≥ 15.0 µg·L⁻¹, oxygen consumption was depressed throughout the exposure period.

Four out of five pre-exposure values for treatment groups were significantly lower than the control pre-exposure values (Table 4). This intergroup variability is reflected also in the finding that the majority of the post-exposure values for treatment groups were lower than the corresponding post-exposure control values.

Hematological Analyses:

Significant elevations in plasma lactate were observed in all treatment groups exposed at and above 7.5 µg·L⁻¹ TCMTB (Figure 13). Lactate levels reached a maximum (5.29 mM·L⁻¹, SEM=0.55) within the 20.0 µg·L⁻¹ treatment group. Plasma lactate levels of the carrier-exposed fish were determined at
TABLE 4. Oxygen consumption (MO$_2$) rates (mg O$_2$·kg$^{-1}$·h$^{-1}$) for juvenile coho salmon prior to and during exposure to TCMTB.

<table>
<thead>
<tr>
<th>TCMTB Concentration (µg·L$^{-1}$)</th>
<th>Control</th>
<th>5.0</th>
<th>7.5</th>
<th>10.0</th>
<th>15.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Exposure (24-48 h)</td>
<td>92.34</td>
<td>80.76$^b$</td>
<td>83.52$^b$</td>
<td>76.08$^b$</td>
<td>82.14$^b$</td>
<td>97.56</td>
</tr>
<tr>
<td></td>
<td>(2.19)</td>
<td>(1.92)</td>
<td>(2.23)</td>
<td>(1.74)</td>
<td>(2.67)</td>
<td>(2.62)</td>
</tr>
<tr>
<td>Post-Exposure (0-24 h)</td>
<td>89.70</td>
<td>75.06$^{ab}$</td>
<td>79.38$^b$</td>
<td>73.68$^b$</td>
<td>77.22$^b$</td>
<td>83.94$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>(1.97)</td>
<td>(2.11)</td>
<td>(2.31)</td>
<td>(1.93)</td>
<td>(2.56)</td>
<td>(1.97)</td>
</tr>
<tr>
<td>Post-Exposure (24-48 h)</td>
<td>95.46</td>
<td>92.38$^{ac}$</td>
<td>76.62$^{ab}$</td>
<td>78.60$^b$</td>
<td>70.86$^{abc}$</td>
<td>84.30$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>(2.39)</td>
<td>(3.67)</td>
<td>(2.60)</td>
<td>(2.40)</td>
<td>(1.96)</td>
<td>(2.34)</td>
</tr>
</tbody>
</table>

$^a$ Significant difference in comparison with own pre-exposure MO$_2$ value (P < 0.05, paired Student's t-test).

$^b$ Significant difference in comparison with corresponding control MO$_2$ value (P < 0.05, unpaired Student's t-test).

$^c$ Significant difference in comparison to own MO$_2$ value for preceding (0-24 h) post-exposure period (P < 0.05, paired Student's t-test).

Note: Values are presented as mean (SEM). The number of experimental replicates was 5 (except at 15.0 µg·L$^{-1}$ where n=4), with each replicate representing 48 single MO$_2$ measurements for 4/5 pooled fish.
1.37 mM·L⁻¹, slightly lower than those of the controls (1.62 mM·L⁻¹, SEM=0.07).

An apparent, but not significant increase in plasma glucose was observed to coincide with increasing TCMTB concentrations (Figure 14). Plasma glucose levels ranged from 3.27 mM·L⁻¹ (SEM=0.56) for the 20.0 μg·L⁻¹ treatment group down to 2.02 mM·L⁻¹ (SEM=0.19) for the controls. At 2.60 mM·L⁻¹ the carrier fish exhibited slightly, but not significantly, higher glucose levels than the controls.

Hematocrit values appeared to increase at TCMTB concentrations ≥7.5 μg·L⁻¹, though this effect was also not significant (Figure 15). Control hematocrits at 37.8% (SEM=1.0) were only slightly lower than those recorded for the 15.0 and 20.0 μg·L⁻¹ treatment groups, both at 39.4% (SEM=1.1 and 1.0, respectively).

Significant reductions in leucocrit values were observed in all treatment groups exposed to ≥15.0 μg·L⁻¹ (Figure 16). Leucocrit reached a minimum of 0.78% (SEM=0.04) with the 20.0 μg·L⁻¹ treatment group. Leucocrits of the carrier exposed fish (0.95%, SEM=0.10) were not significantly lower than those of the control fish (1.02%, SEM=0.05).
FIG. 13. Mean plasma lactate concentrations in juvenile coho salmon (Oncorhynchus kisutch) following 48 h of exposure to various TCMTB concentrations. N = 5 replicates, with plasma pooled from 5 individual fish for each replicate. Vertical bars indicate SEM.
* Significant difference from control (P<0.05)

FIG. 14. Mean plasma glucose concentrations in juvenile coho salmon (Oncorhynchus kisutch) following 48 h of exposure to various TCMTB concentrations. N = 5 replicates, with plasma pooled from 5 individual fish for each replicate. Vertical bars indicate SEM.
FIG. 15. Mean hematocrit values for juvenile coho salmon (*Oncorhynchus kisutch*) following 48 h of exposure to various TCMTB concentrations. N=25. Vertical bars indicate SEM.

FIG. 16. Mean leucocrit values for juvenile coho salmon (*Oncorhynchus kisutch*) following 48 h of exposure to various TCMTB concentrations. N=25. Vertical bars indicate SEM.

* Significant difference from control (P<0.05)
Gill Histology:

The gill morphometry results obtained from the juvenile chinook salmon removed during the acute lethal bioassays are summarized in Table 5. The structure of the secondary lamellae in the control gills was similar to that described by Skidmore and Tovell (1972) (Figure 17a). Interlamellar distances and blood-water diffusion distances of control fish were 39.56 μm (SEM=0.89) and 6.98 μm (SEM=1.75), respectively. Separation of the lamellar epithelium was infrequently observed in control fish and when present was minimal.

After exposure to between 6.0 to 10.0 μg·L⁻¹ TCMTB (exposure period of 49.5 to 146 h), separation of the lamellar epithelium was pronounced in most gills examined. Hypertrophy of epithelial cells around the distal surfaces of gill lamellae and a slight curling of the lamellae themselves was also evident (Figure 17b). Distances between adjacent lamellae were reduced by ca. 27 to 41% from controls, whereas blood-water diffusion distances increased by 43 to 193% over control values. Decreases in interlamellar distance were statistically significant at 6.0 μg·L⁻¹ but not at 10.0 μg·L⁻¹. Significant increases in blood-water diffusion distance occurred at 10.0 μg·L⁻¹ but not at 6.0 μg·L⁻¹.

Hypertrophy of epithelial cells, curling of lamellae and detachment of the epithelium were prominent in gills taken from fish exposed to 20.0 μg·L⁻¹ TCMTB (exposure period of 49.0 to 72.3 h). Obliteration of the interlamellar
channels and fusion of adjacent lamellae were noted in a number of the sections examined. In addition, a complete detachment of the lamellar epithelium was evident at the distal end of filaments in fish receiving the longest exposure periods. At this point interlamellar distances had decreased significantly by 74% and blood-water diffusion distances increased significantly by 276% compared to control fish (Figure 17c).

After less than 46 h exposure, fish exposed to 40.0 μg·L⁻¹ TCMTB demonstrated symptoms similar to those of the 20.0 μg·L⁻¹ treatment group. Fusion of adjacent secondary lamellae had become slightly more pronounced (Figure 17d). Similar to the 20.0 μg·L⁻¹ treatment group, interlamellar distances (74% decrease) and blood-water diffusion distances (265% increase) were significantly altered from control fish values.

Figures 18 and 19 depict the effects of both exposure duration and concentration on interlamellar and blood-water diffusion distances, respectively. Interlamellar and blood-water diffusion distances of control fish remained above 37.98 μm and below 10.34 μm, respectively, over all exposure durations examined. At concentrations ≤6.0 μg·L⁻¹ neither interlamellar distance nor blood-water diffusion distance appear to be dependent on the length of the TCMTB exposure. Concentrations equal to and greater than 20.0 μg·L⁻¹ TCMTB evoked similar responses in terms of gill morphometric parameters, at the two shorter exposure periods (0-54 h and 55-76 h). At 20.0 μg·L⁻¹ and above, interlamellar distance remained above 5.46 μm and blood-water diffusion distance remained below 31.74 μm,
regardless of increasing exposure duration or concentration. Since no fish survived concentrations greater than 20.0 μg·L⁻¹ for more than ca. 72 h, gill responses were not examined after this time period. The effects of variable exposure times were most pronounced at concentrations between 6.0 to 20.0 μg·L⁻¹. Here, short exposure periods lessened the deleterious effects on gill structure, while longer exposures exacerbated the effects. The interlamellar distances recorded for fish exposed to 10.0 μg·L⁻¹ for periods ≤54 h are ca. three times greater than those of fish exposed to the same concentration for periods ≥76 h.
TABLE 5. Effect of TCMTB on gill morphology: Interlamellar and blood-water diffusion distances in gills of TCMTB-treated chinook salmon (*Oncorhynchus tshawytscha*).

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>Measured Parameters (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCMTB Conc. (µg L⁻¹)</td>
<td>Exposure Duration (h)</td>
</tr>
<tr>
<td>0.0</td>
<td>0-146</td>
</tr>
<tr>
<td>6.0</td>
<td>55-76</td>
</tr>
<tr>
<td>6.0</td>
<td>77-146</td>
</tr>
<tr>
<td><strong>6.0</strong></td>
<td><strong>0-146</strong></td>
</tr>
<tr>
<td>10.0</td>
<td>0-54</td>
</tr>
<tr>
<td>10.0</td>
<td>55-76</td>
</tr>
<tr>
<td>10.0</td>
<td>77-146</td>
</tr>
<tr>
<td><strong>10.0</strong></td>
<td><strong>0-146</strong></td>
</tr>
<tr>
<td>20.0</td>
<td>0-54</td>
</tr>
<tr>
<td>20.0</td>
<td>55-76</td>
</tr>
<tr>
<td><strong>20.0</strong></td>
<td><strong>0-146</strong></td>
</tr>
<tr>
<td>40.0</td>
<td>0-146</td>
</tr>
</tbody>
</table>

* Significantly different from control mean (p<0.05)

Note: Values in bold print represent results for the total number of fish examined at any given concentration. n = total number of fish examined.
FIG. 17a. Photomicrograph of a transverse section through a gill filament from a control chinook salmon (*Oncorhynchus tshawytscha*). Wax-embedded, 7 µm section stained with hematoxylin and eosin (X400).

FIG. 17b. Photomicrograph of a transverse section through a gill filament from a 10.0 µg·L⁻¹ TCMTB-treated chinook salmon (*Oncorhynchus tshawytscha*) which had reached the stage of overturn. Wax-embedded, 7 µm section stained with hematoxylin and eosin (X400).
FIG. 17c. Photomicrograph of a transverse section through a gill filament from a 20.0 μg.L⁻¹ TCMTB-treated chinook salmon (*Oncorhynchus tshawytscha*) which had reached the stage of overturn. Wax-embedded, 7 μm section stained with hematoxylin and eosin (X400).

FIG. 17d. Photomicrograph of a transverse section through a gill filament from a 40.0 μg.L⁻¹ TCMTB-treated chinook salmon (*Oncorhynchus tshawytscha*) which had reached the stage of overturn. Wax-embedded, 7 μm section stained with hematoxylin and eosin (X400).
FIG. 18. Effect of TCMTB on gill histology: Measured interlamellar distances in juvenile chinook salmon (*Oncorhynchus tshawytscha*) in relation to duration and concentration of TCMTB exposure. See Table 5 for exact details on numbers of individuals sampled for each point.

FIG. 19. Effect of TCMTB on gill histology: Measured blood-water diffusion distances in juvenile chinook salmon (*Oncorhynchus tshawytscha*) in relation to duration and concentration of TCMTB exposure. See Table 5 for exact details on numbers of individuals sampled for each point.
Discussion:

Acute, Lethal Bioassays:

The mean 96-h LC$_{50}$ value of 10.8 µg·L$^{-1}$ provides basic information on the acute toxicity of TCMTB to juvenile chinook salmon. Values of the 96-h LC$_{50}$ for chinook salmon obtained here are similar but slightly lower than those reported for other salmonid species; coho salmon at 17.3 µg·L$^{-1}$ (G. Kruzynski, pers. comm.) and rainbow trout at 49.5 µg·L$^{-1}$ (Ward 1989). The higher LC$_{50}$ value reported for rainbow trout may be due to an inherent underestimate of toxicity in static bioassays.

Swimming Performance:

Reductions in critical swimming speed occurred in a concentration dependent manner and were significant at sublethal concentrations of 10.0 µg·L$^{-1}$ (ca. 60% of the 96-h LC$_{50}$), 5.0 µg·L$^{-1}$ (ca. 45% of the 96-h LC$_{50}$) and 10.0 µg·L$^{-1}$ (ca. 20% of the 96-h LC$_{50}$) for coho, chinook and rainbow trout, respectively.

The control values reported in this study suggest that the test fish were sufficiently acclimated to the experimental apparatus. The mean critical swimming speeds of 6.29 BL·s$^{-1}$ for coho controls are similar to the
5.91 BL·s⁻¹ value reported by Howard (1975) for fingerling coho under comparable conditions. Likewise, critical swimming speeds of control chinook and rainbow trout, at 5.98 and 5.27 BL·s⁻¹ respectively, are comparable to values reported by other authors (5.3 and 5.5-6.4 BL·s⁻¹) for fish of a similar size (Davis et al. 1963, Fry and Cox 1970, respectively).

Reductions in critical swimming speed are often related to impaired transport or exchange of respiratory gases (Sprague 1971, Satchell 1984). Jones (1971) demonstrated reduced swimming performance under hypoxic (low environmental oxygen tension) and anaemic (low blood oxygen capacity) conditions. He suggested several factors which may be involved in limiting performance at critical speeds; inability to supply enough oxygen to the gills, inability to deliver enough oxygen to the tissues, inability to remove metabolic products or to provide adequate substrate or to activate enzymatic processes. The observed reductions in critical swimming speed reflect a possible limitation in one or more of these functions. Heath (1987) postulated that critical swimming speed is particularly sensitive to impairment of oxygen transfer across the gill. Reduced aerobic performance has been demonstrated in cases where toxicants bind or adsorb to gill epithelium (Howard 1975), clog lamellae (MacLeod and Smith 1966) and cause structural gill damage at the microscopic level (Waiwood and Beamish 1978). As disturbed gill integrity, specifically lamellar clubbing, has been previously observed in chinook salmon after sublethal exposures to TCMTB (G. Kruzynski, pers. comm.) it is probable that the reductions in critical swimming speed may represent a limitation in maximum oxygen transfer efficiency resulting from TCMTB-associated gill damage.
Previous investigations have implicated lamellar injury as a possible barrier to efficient gas exchange in fish sublethally exposed to toxicants (Skidmore 1970, Skidmore and Tovell 1972). Hypertrophy and desquamation of the gill epithelium act to decrease the total lamellar surface area available for gas exchange and to increase the diffusion distances for oxygen and/or carbon dioxide transfer (Hughes et al. 1979). Development of either of these features in response to sublethal toxicant exposure will therefore reduce the efficiency of gas exchange, as well as affecting extra-renal excretion and ionic exchange at the gills. Epithelial hypertrophy and lifting was prominent among fish exposed to TCMTB, indicating that occlusion of the interlamellar water space and broadening of the diffusion distance at least partially reduced the efficiency of gas exchange.

The inhibitory effect of TCMTB on swimming performance was also found to be related to the length of exposure; with increased exposure times generally associated with a greater inhibitory effect. Under acute, sublethal situations exposure periods of less than 12 h had no effect on swimming performance, regardless of concentration tested (5 - 40 µg·L⁻¹). Similarly, at concentrations approaching the 96-h LC₅₀ value, extending the exposure period from 24 to 48 h did little to further reduce swimming performance. In terms of the critical swimming speed, therefore, the 12 to 24 h period of exposure is most critical in salmonids exposed to sublethal TCMTB concentrations.
Studies with coho salmon exposed to sublethal concentrations of bleached kraft pulpmill effluent (BKME) demonstrated a similar effect (Howard 1975). Critical swimming speeds were reduced at BKME concentrations equivalent to 20% of the 96-h LC₅₀ or greater after an 18-h exposure. In this investigation it was determined that extending the exposure period from 18 to 168 h caused no further reductions in swimming performance for any single concentration of BKME. Also, at shorter exposure times the differences due to concentration were less clear. The Howard (1975) study and the present work imply that the physiological causes behind interference with critical performance occur within a distinct exposure interval. If it is assumed that oxygen uptake and delivery are the factors limiting performance then this impairment of gas transfer must also occur within a similar, distinct exposure interval. Based on these observations, further studies concerning the effects of TCMTB on swimming performance should be limited to a maximum exposure time of 24 h. No further exacerbation of the effect occurred at exposures exceeding this duration.

**Oxygen Consumption Rate:**

Mean daily oxygen consumption rates (97 mg O₂·kg⁻¹·h⁻¹) for control fish fall between resting values of 95 and 101 mg O₂·kg⁻¹·h⁻¹ as reported by Brett and Zala (1975) and Barton and Schreck (1987), respectively, for juvenile salmonids at similar temperatures. The early morning oxygen consumption increases and nightly decreases reflect normal daily activity patterns (Brett and Zala 1975). A comparison with other published standard (or basal) and routine oxygen uptake rates is presented in Table 6. Routine oxygen
TABLE 6. Rates of oxygen uptake of salmonids at approximately 15 °C.

<table>
<thead>
<tr>
<th>Species</th>
<th>Weight (g)</th>
<th>Rate of O₂ Uptake (mg O₂/kg fish/h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brook Trout</td>
<td>200</td>
<td>98, S*</td>
<td>Beamish (1964)</td>
</tr>
<tr>
<td>Rainbow Trout</td>
<td>210±60</td>
<td>95, R*</td>
<td>Skidmore (1970)</td>
</tr>
<tr>
<td>Sockeye Salmon</td>
<td>200</td>
<td>71, S</td>
<td>Brett (1965)</td>
</tr>
<tr>
<td>Coho Salmon</td>
<td>6</td>
<td>135, S</td>
<td>Johansen and Geen (1990)</td>
</tr>
<tr>
<td>Coho Salmon</td>
<td>11</td>
<td>120, R</td>
<td>Janz et al. (1991)</td>
</tr>
<tr>
<td>Coho Salmon</td>
<td>25</td>
<td>97, R</td>
<td>This Paper</td>
</tr>
</tbody>
</table>

*S = Standard rate of oxygen uptake
*R = Routine rate
consumption or metabolic rate is thought to be a good approximation of the standard or basal metabolic rate when the fish is relatively inactive and the exposure chamber is small (Heath 1987). Therefore, in this study it can be reasonably assumed that the measured routine oxygen consumption rates approached the standard rate.

Oxygen consumption rates, although altered, did not respond to TCMTB in a predictable concentration-dependent manner as did swimming speeds. A consistent and significant reduction in oxygen consumption rates over the entire exposure period occurred only in the 20.0 µg·L⁻¹ treatment group (ca. 120% of the 96-h LC₅₀ value). However, significant alterations in oxygen consumption were apparent at even the lowest concentration tested (ca. 30% of the 96-h LC₅₀ value). The extent of the intergroup variability made it difficult to separate the regular variability in oxygen consumption rates from the overall response to the toxicant. Extending the acclimation period further in an attempt to lessen this variability may have led to starvation-related artifacts in the oxygen consumption data. Excessive food deprivation is known to cause abnormally low rates of metabolism in fish (Beamish 1964).

Most kinds of stress stimuli elicit a response of oxygen uptake (Kaufmann et al. 1989). The response is usually non-specific, but whenever a toxic agent damages the respiratory epithelia of the animal, the degree of respiratory distress is an indicator of stress intensity (Hughes 1981). Generally, elevated oxygen consumption is a reflection of increased spontaneous locomotor
activity caused by irritation of skin or mucus membranes (Heath 1987). Hyperactivity-associated increases in oxygen consumption have been implicated in sublethal organochlorine and zinc toxicity (Waiwood and Johansen 1974, Brafield and Mathiesson 1976). The neurotoxic actions of certain chemicals can make a fish hypoactive as well as hyperactive, thus lowering the standard oxygen consumption rate (Rao et al. 1985). Other researchers have found the standard oxygen consumption rate to be relatively unaffected by toxicants. Skidmore (1970) found that the rate of routine oxygen uptake in fish exposed to a rapidly lethal solution of zinc sulphate remained unchanged until 80% of the survival time had elapsed, and then declined rapidly. The slight reductions in oxygen consumption noted with some of the TCMTB concentrations are suggestive of a hypoactive rather than hyperactive state.

It may also be possible that TCMTB is able to exert a greater inhibitory effect on active oxygen consumption rates. MacLeod and Smith (1966) reported that suspensions of conifer wood fibers depressed the active oxygen consumption rates of fathead minnows, Pimphales promelas. Their suggested mechanism whereby active metabolism is decreased by wood fiber was related to a decreased efficiency of the respiratory system due to a clogging of the gill structure with fiber and interruption of respiratory flow during gill-cleaning reflexes. Although not measured here, TCMTB may have a similar limiting effect on oxygen uptake during more demanding activity. The reduced critical swimming speed observed in this study suggests an inability to satisfy active oxygen requirements. Additionally, Heath (1987) suggested that the amount of respiratory impairment which would be
unlikely to affect a fish's ability to function in the environment at rest will undoubtedly have an inhibitory effect on active performance. In this regard, it would seem worthwhile to conduct measurement of both routine and active oxygen consumption when studying respiratory function effects, particularly where the mode of toxicant action is poorly understood.

In the present study, routine oxygen consumption was relatively insensitive when effect thresholds are compared to the other measured parameters. The variability in oxygen consumption rates between untreated groups were, in some cases, greater than the overall response to the toxicant itself. The absence of a concentration-dependent response to the toxicant was another notable drawback of this technique.

**Hematology and Blood Chemistry:**

The results indicate that plasma lactate levels rose significantly with concomitant increases in TCMTB concentration. Lactate elevations exhibited an effect threshold of 7.5 μg·L⁻¹ (ca. 40% of the 96-h LC₅₀ value) and followed a concentration dependent fashion.

Plasma lactate control values of 1.62 mM·L⁻¹ are comparable to those reported by other authors (1.88 - 2.23 mM·L⁻¹ by Janz et al. 1991; 1.49 mM·L⁻¹ by Andersson et al. 1988) for fish of similar size, but higher than those found in larger, cannulated fish (0.71 mM·L⁻¹ by Wood et al. 1988). Where the size of the fish makes cannulation impractical, blood must be
sampled from the caudal vasculature via the severed caudal peduncle. Like plasma lactate, muscle lactate increases in response to stress but attains much higher concentrations than in the plasma (Milligan and Wood 1986). Thus, although contact between blood and tissue was minimized, a small addition of muscle lactate to the blood was likely unavoidable during the sampling process. This may have resulted in a systematic error which overestimates total plasma lactate, but will not have affected the overall conclusion that elevated plasma lactate levels imply stressed fish. Alternatively, it may be argued that stress associated with the sampling process introduces another systematic error which may overestimate total plasma lactate. Such an error would be expected to lower the sensitivity of this technique. However, there is good agreement between the threshold for the lactate response and several of the other parameters examined here. It therefore seems unlikely that sampling-associated biases are contributing to the results.

Elevations in the concentration of plasma lactate can be indicative of anaerobic metabolism often associated with acute physiological stress (Heath 1987). Anaerobic metabolism and subsequent increases in lactate might be expected if an impairment of oxygen flux from the water to the blood was occurring under TCMTB exposure. However, it can not be determined whether the observed increases in plasma lactate were due to either stress-related adrenergic stimulation of glycolysis (including toxicant avoidance behaviour, hyperactivity, etc.) or the direct effect of the toxicant-induced hypoxia on muscle tissues. Under the former situation the presence of TCMTB could elicit a generalized stress response, triggering a release of catecholamines into the blood. Glycolysis, and subsequently plasma lactate,
have been shown to increase in response to elevated catecholamine levels (Mazeaud et al. 1977). In the latter case, TCMTB-induced impairment of oxygen transfer to the tissues may have been reflected in a greater proportion of the metabolic demand for ATP being supplied through anaerobic as well as aerobic processes.

As an indicator of acute physiological stress, plasma lactate is responsive to a variety of extraneous disturbances, including handling (Wedemeyer 1972). Caution must be taken to separate the handling stress from that of the toxicant. Tissue injury during sample collection may also lead to non-toxicant related elevations in plasma lactate levels.

Elevation of plasma glucose levels in fish exposed to sublethal concentrations of TCMTB were indicated, but were not statistically significant. Control values at 2.02 mM·L⁻¹ were similar to those reported by Janz et al. (1991) at 2.53-3.12 mM·L⁻¹, Casillas and Smith (1977) at 2.6-3.6 mM·L⁻¹ and Silbergeld (1974) at 2.09-3.31 mM·L⁻¹ for juvenile coho, adult rainbow trout and johnny darters (Etheostoma nigrum), respectively. Barton et al. (1980) and Redding and Schreck (1987) have demonstrated increased plasma glucose concentrations up to 11.34 mM·L⁻¹ in fish subjected to handling stress and suspended solids, respectively.

The rapid increases in concentrations of circulating catecholamines or corticosteroids which occur following a toxicant-induced disturbance are known to increase circulating glucose levels within a matter of minutes (Nakano and Tomlinson 1967). However, when a fish enters the stage of
physiological resistance following the initial alarm reaction, plasma glucose levels may return to normal or remain only slightly elevated (Mazeaud et al. 1977). McLeay (1977) found that the hyperglycemic stress response of juvenile coho salmon exposed to pulpmill effluent was most pronounced after 3-6 h exposure and had returned to near basal levels by 24 h. If the hyperglycemic response of the coho used in this study was to follow a similar pattern, the length of the exposure period (48 h) may have reduced the possibility of detecting an earlier glucose peak. However, Silbergeld (1974) was able to detect significant glucose elevations in johnny darters for up to 15 days of exposure to the insecticide, dieldrin. The effect of these more prolonged exposures to toxicants on the plasma glucose response is thus less defined.

Some evidence suggests (Nakano and Tomlinson 1967) that the magnitude and duration of the hyperglycemic response in fish, as in mammals, is greater in those with high liver glycogen reserves. McLeay (1977) found that coho acclimated for 72-96 h to pulpmill effluent did not demonstrate the same degree of hyperglycemia as those acclimated for only 24-48 h. Black et al. (1966) found a 62% reduction in liver glycogen reserves of rainbow trout after 84 h of starvation. In these studies, it was presumed that starvation during the extended acclimation period reduced carbohydrate energy reserves resulting in a diminished hyperglycemic response. The 120 h period (24 h acclimation, 48 h pre-exposure and 48 h exposure) preceding glucose sampling used here may have resulted in starvation-related interferences with the measurable hyperglycemic response. The known influence of diet, time since last feeding, state of liver glycogen stores, fish developmental stage and season on the magnitude of the
hyperglycemia caused by the stressor necessitates consideration of these variables when monitoring the plasma glucose level as an index of stress (McLeay 1977).

It has been suggested (Morales et al. 1990) that anaesthesia use during fish handling and sampling procedures may actually act as a stress inducer. However, Puceat et al. (1989) have demonstrated that the anaesthetic 2-phenoxyethanol inhibited glucose release in isolated rainbow trout hepatocytes after less than 2 minutes exposure. It is conceivable that a 2-phenoxyethanol mediated inhibition of glycogenolysis may have also been involved in the low level of hyperglycemic response noted in the TCMTB-exposed coho.

Previous investigations suggest that a shorter toxicant exposure time may improve the sensitivity of the glucose assay, since plasma glucose concentrations tend to return to basal levels after initial toxicant-induced increases (McLeay 1977). However, even if shorter exposure times were to elicit a significant glucose response, other disadvantages of this test must also be considered. The extent of plasma glucose elevation appears to be affected by the glycogen energy stores of the fish which are in turn dependent on a number of physiological and environmental factors (Nakano and Tomlinson 1967). Another drawback is that a hyperglycemic response caused by increased secretions of corticosteroid and catecholamine stress hormones is evoked by many other stressors (Wedemeyer and McLeay 1981). Therefore, the test is susceptible to extraneous disturbances and must be performed under strictly controlled conditions. Finally, as with
plasma lactate, the biological significance of increased plasma glucose levels can not be easily interpreted.

In the same manner as plasma glucose, measurement of plasma lactate, cortisol and catecholamine levels can be used to indicate stress-related neurohormonal imbalances. Plasma lactate however, appears to be better suited to TCMTB studies since it is more reliable than plasma glucose and less costly than either of the latter two techniques.

In this study a slight, but statistically insignificant increase in hematocrit was observed to coincide with a proportional increase in TCMTB concentration. Control values at 37.8% were higher than those values reported by Janz et al. (1991) at 29-32% and McLeay and Gordon (1977) at 24-28% for juvenile coho reared under similar conditions. Normal hematocrit values for healthy juvenile rainbow trout are in the range of 24-43% (Wedemeyer and Yasutake 1977). Stress-mediated alterations in hematocrit are known to occur either through a release of erythrocytes from storage organs, a swelling of erythrocytes themselves, or plasma volume changes associated with gill damage (McLeay and Gordon 1977). Although hematocrit and red-blood cell counts have been suggested as measurable sublethal indicators (Wedemeyer and Yasutake 1977), other authors were also unable to demonstrate hematocrit changes at concentrations of toxicants which evoked other stress-related changes (Iwama et al. 1976, Janz et al. 1991). Sprague (1971) noted that the measurement of hematocrit gives a variable response which may be less sensitive than survival, growth or histopathology as an indicator of sublethal toxicity.
Reductions in leucocrit occurred parallel to an increase in TCMTB concentration with an observed threshold of 15.0 μg·L⁻¹. Control values at 1.02% are similar to those reported by McLeay and Gordon (1977) at 1.11% and Janz et al. (1991) at 1.01 to 1.23% for juvenile coho reared under similar conditions. Acute exposure to sublethal concentrations of kraft pulpmill effluent has been shown repeatedly to depress white blood cell (WBC) counts in juvenile coho salmon (McLeay 1975). Similarly, short-term exposures of these species to other toxicants, including dehydroabietic acid, at concentrations which did not alter erythrocyte counts or hematocrit values appreciably, caused marked decreases in WBC counts (Iwama et al. 1976).

These decreases in WBC counts and the present depressions of leucocrit values due to TCMTB are consistent with the stress-mediated hematological response. Reductions in numbers of circulating leucocytes (predominantly small lymphocytes) and thrombocytes appear to typify these species' hematological responses to increased levels of circulating pituitary and corticosteroid stress hormones (McLeay and Gordon 1977). The lymphocytolytic properties of the corticosteroids may serve to immediately increase the available antibody titer and provide a ready source of protein for gluconeogenesis (Iwama et al. 1976). Unfortunately, this secondary stress response also has deleterious side-effects in terms of depression of the immunological system and loss of resistance to infectious diseases.
Gill Histology:

Extensive gill injury was evident among chinook salmon exposed to sublethal and lethal concentrations of TCMTB but was most severe at exposure concentrations $\geq 10.0 \mu g \cdot L^{-1}$. Quantitative measurements of gill injury demonstrated significant changes to lamellar morphology down to $6.0 \mu g \cdot L^{-1}$. A maximal effect concentration of $20.0 \mu g \cdot L^{-1}$ was also indicated. Exposures exceeding this concentration imparted no further reductions in interlamellar space or increases in blood-water diffusion distances, regardless of the length of the exposure used in this study. This suggests that an upper limit of 31.74 $\mu m$ for blood-water diffusion distance and a lower limit of 5.46 $\mu m$ for interlamellar distance constitute a threshold beyond which the survival of the fish was adversely affected. Damage appeared to be dependent on the duration, as well as the concentration of the exposure for the concentration range between $6.0 - 20.0 \mu g \cdot L^{-1}$. Mean interlamellar distances of control fish (39.56 $\mu m$) are in agreement with those reported by Moyle and Cech (1982) for rainbow trout (35-40 $\mu m$). Blood-water diffusion distances of 6.98 $\mu m$ for control fish are also similar to values of 3 - 6.7 $\mu m$ reported for healthy rainbow trout (Moyle and Cech 1982, Satchell 1984).

A slight separation of the epithelium, as was noted in a number of control gills from this study, is a commonly reported occurrence in gill histopathology studies (Mallatt 1985). Speare and Ferguson (1989) concluded that the presence of slight lamellar epithelial lifting in healthy rainbow trout gills was indicative of preparation and postmortem artifacts rather than a pathological lesion. Air exposure during postmortem dissection was believed to be the major contributor to this artifact, which
occurred even when tissue fixation commenced as soon as 20 s after death. Standardization of tissue processing in control and treatment fish was deemed necessary to remove biases associated with fixation delays (Speare and Ferguson 1989). As the postmortem technique utilized in this study attempted to use a standard and relatively short fixation delay, any artifact-related biases between the various groups should be minimal.

The alteration of lamellar structure in response to TCMTB exposure follows the typical chronology of damage from acute exposure with other toxicants outlined by Heath (1987). He noted that initial exposure resulted in a lifting of the outer layer of the lamellar epithelium (desquamation) and formation of edematous spaces between the epithelial layers. With continued exposure this was eventually followed by a detachment of the whole epithelium and a resultant loss in lamellar rigidity.

It should be emphasized that the present histology study was undertaken as a complement to the mortality bioassays and as such was constrained by the bioassay’s requirements. Gills, for instance, could only be removed from fish as they became moribund or from survivors at the termination of the experiment. Thus, the exposure durations examined were determined by the acute toxicity of the TCMTB concentrations to which the fish were exposed. Sacrificing fish at regular exposure, as well as concentration intervals may have provided more extensive information on the function of TCMTB in gill injury. Due to the comparatively low numbers of surviving fish examined, it is unclear from this exploratory study what effect sublethal concentrations of TCMTB have on the gill prior to the fish reaching a
moribund state. However, data from surviving fish tends to suggest that the observed gill injury occurs as a gradual process.
Summary and Conclusions:

The increased leucocrit, plasma lactate and evidence of gill histopathology indicate a physiological stress response to acute, sublethal TCMTB exposure. The perturbations in gill structure and associated reduction in critical swim speed suggest that impaired respiratory function may be a significant contributing factor to this stress response. Alterations in lamellar structure can contribute to impaired gas exchange capabilities at the water/blood barrier in the gills of fish. The imposed stress resulting from this impaired gill function could act to limit critical performance by restricting oxygen supply to the swimming muscles and depleting anaerobic reserves during submaximal swimming. The overall implication of the above is that a reduction of swimming performance occurred at least partly as a result of TCMTB-induced alteration of gill structure. The impairment of respiratory function is not reflected in the measured routine oxygen consumption rates. The absence of a measurable, concentration-dependent alteration in resting oxygen consumption rates may indicate that this parameter was not as sensitive to disturbance of gill integrity as was critical swimming performance. It may, however, indicate that the impaired gill function only becomes a limiting factor during periods of intense activity, as occurs during swimming at critical speeds.

The lower critical swimming performance observed may represent a stress reaction mounted in response to some other effect of acute, sublethal TCMTB exposure. Increased plasma lactate levels are indicative of a greater anaerobic energy production and could be related to hyperactivity, stress, or
be a result of reduced tissue oxygen levels due to gill damage. The observed reduction in leucocrit simply confirms that TCMTB elicits a significant generalized stress response in salmonids.

The parameters measured in this study vary with respect to their usefulness as indicators of sublethal toxicity. Swimming speed, plasma lactate, leucocrit and gill histopathology were the techniques most sensitive to the sublethal effects of the concentrations of TCMTB employed in this study. All four parameters are generally in good agreement with one another regarding the minimum TCMTB concentration range (6 to 15 µg·L⁻¹) required to elicit a significant response. Effects of TCMTB on other parameters, such as plasma glucose, hematocrit and routine oxygen consumption were minimal under the conditions of this study.

Although determination of plasma lactate is a rapid, straight-forward and highly sensitive approach to measuring toxicant-induced stress, it does appear to have limitations. Moreover, while plasma lactate may be a fairly sensitive indicator, the biological significance of increased lactate levels is not well understood.

Leucocrit measurement is a similarly, straight-forward and inexpensive technique. However, it was only sensitive at concentrations of TCMTB which were nearing the 96-h LC₅₀ value. The simple nature of this assay and its potential biological implications in terms of immunologic defense mechanisms, merit its use as an indicator of acute, sublethal toxicity.
While histology provides a useful tool for determining the mode of action of a toxicant, quantitating histological changes is tedious and labour-intensive, precluding its routine use as a rapid, acute sublethal test. Furthermore, toxicant-induced gill lesions are generally non-specific and may simply represent a stereotyped physiological reaction to environmental stressors (Mallatt 1985).

Measurement of swimming performance appeared to be one of the more sensitive techniques, with initial alterations occurring at 20-60% of the 96-h LC$_{50}$ value, depending on the particular species examined. In contrast to some of the physiological and hematological measures, impairment of swimming performance is also an ecologically relevant response to a toxicant, particularly for upstream migrating fish. Obviously, this test in its present form was not designed for routine monitoring of toxic-effect concentrations. However, it could be readily adapted for such a purpose. A modified version of the swimming apparatus used in this study could prove useful for in situ examination of the effects of sublethal toxicant exposures on swimming performance.

It has been demonstrated in this study that acute, sublethal exposure of juvenile salmonids to the antisapstain chemical, TCMTB, may have adverse physiological consequences, even at very low exposure levels. With increasingly stringent discharge regulations for TCMTB, it is possible that the forest products industry may switch to other antisapstain agents. From a pro-active perspective, it is therefore necessary to establish meaningful protocols for study requirements prior to registering new compounds for
antisapstain use. The present study has identified some indices that provide useful and meaningful tools to make such assessments.
References


