

# FRED Reports

A FLUOROMETRIC METHOD FOR THE QUANTITATIVE  
DETERMINATION OF SUB-MICROGRAM AMOUNTS  
OF OXYTETRACYCLINE IN SOCKEYE SALMON  
(*Oncorhynchus nerka*) FRY

BY

J. P. Koenings  
Joshua Lipton  
Number 20



**Alaska Department of Fish & Game**  
Division of Fisheries Rehabilitation,  
Enhancement and Development

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Alaska Department of Fish and Game  
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Don W. Collinsworth  
Commissioner

Stanley A. Moberly  
Director

P.O. Box 3-2000  
Juneau, Alaska 99802

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## ABSTRACT

A fluorometric procedure was developed to quantify sub-microgram concentrations of the antibiotic oxytetracycline (OTC) in sockeye salmon (*Oncorhynchus nerka*) fry. The method involves the extraction of oxytetracycline residualized within the skeleton of fry fed OTC-medicated food, and the subsequent measurement of the fluorescent OTC-calcium complex. A linear relationship was found to exist between fluorescence intensity and OTC concentration in both pure samples of OTC standard solution and in samples containing biological material. Furthermore, the extraction efficiency of OTC was found to be independent of the concentration of the antibiotic over the range of 0.47  $\mu\text{g}$  OTC to 9.4  $\mu\text{g}$  OTC, while the minimal concentration the method could detect was 0.17  $\mu\text{g}$  OTC/fry. A total of 1.56  $\mu\text{g}$  OTC was recovered from fry (average weight = 0.40 g) fed OTC-medicated food for 29 days. This level increased to an average of 2.49  $\mu\text{g}$  OTC in fry (average weight = 1.0 g) after 60 days of feeding.

## INTRODUCTION

It has been noted by several authors that oxytetracycline (OTC), a broad spectrum antibiotic, may be deposited in the skeleton of hatchery reared salmon fry and fingerlings fed OTC-medicated food (Weber and Ridgeway, 1962, 1967; Weber and Wahle, 1969; Wiltzius, 1980). When exposed to ultraviolet (UV) light, treated fry exhibit varying degrees of a fluorescing yellowish ring within calcified bone especially the spine. Because of the relative ease of applying OTC, efforts have been made to employ this method as a means of identifying hatchery fish (Weber and Ridgeway, 1962, 1967; Weber and Wahle, 1969; Wiltzius, 1980).

However, the current practice of visually identifying fluorescently "marked" fish under UV light is a highly subjective procedure. Furthermore, to generate the level of residualized OTC needed for visual detection of a mark, the fry must be fed heavy doses of OTC, or they must be fed for longer periods until there is more calcified bone deposition. However, increased treatment time, especially in cold water production facilities, is not cost effective when used solely to implement a fry marking program. Thus, a technique was needed to not only detect OTC, but also to determine the shortest rearing period after which detectable levels of OTC could be quantified.

Several procedures have been developed to chemically extract and quantitatively analyze the family of tetracycline antibiotics in biological materials. For example, Kohn (1961) developed a fluorometric technique to extract tetracyclines, Ibsen et al. (1963) described a technique involving the extraction of OTC into amyl alcohol and the formation of a magnesium chelate, and finally, Argauer and Gilliam (1974) developed a fluorometric method for determining OTC in honey bees and pollen patties.

The method developed by Kohn (1961) was found to be suitable for the quantitative detection of tetracycline, but was not suitable for the specific detection of OTC. This anomaly was later corrected by the procedure described by Ibsen, et al. (1963). Using this method, we were able to extract oxytetracycline in sub-microgram concentrations from standard solutions, yet the high level of natural background fluorescence we found when analyzing fry samples made the extraction and quantification of low levels of OTC from fish impossible, and rendered even qualitative determinations of an OTC mark highly problematic. Finally, a modified procedure based on the work of Argauer and Gilliam (1974) was found to be specific for microgram quantities of OTC, and at the same time eliminated the high background fluorescence previously found when analyzing biological samples. The technique described here, a further modification of the Argauer and Gilliam (1974) procedure allowed us to quantitatively detect sub-microgram amounts of residualized OTC in hatchery-fed sockeye salmon (*O. nerka*) fry. This is the first time a fluorometric method has been developed and used to quantify the total body burden of OTC in rearing fry.

Since many hatcheries currently feed OTC-medicated food to fry prior to release (as a preventive measure against disease), the ingestion and subsequent residualization of OTC by large numbers of fry would provide an

economical, non-toxic internal mark. Quantitative recovery of such a mark would enable the clear identification of non-wild fry and thus, allow the in-lake differentiation between hatchery and wild salmon stocks.

## METHODS

### Reagents

Reagent grade trichloroacetic acid (TCA) ( $\text{CCl}_3\text{COOH}$ ) and ammonium hydroxide ( $\text{NH}_4\text{OH}$ ) were used without further purification, and were dispensed from acid-cleaned reagent bottles with Brinkman dispensettes. Ethylacetate ( $\text{CH}_3\text{COOC}_2\text{H}_5$ ), ethylacetoacetate ( $\text{CH}_3\text{COCH}_2\text{COOC}_2\text{H}_5$ ) and calcium chloride dihydrate were Baker analyzed reagents.

### Standards

Standard solutions of Sigma oxytetracycline hydrochloride (potency = 940  $\mu\text{g}/\text{mg}$ ) were prepared in concentrations of 9.4 and 94  $\mu\text{g}/\text{ml}$  in deionized water, and were refrigerated in light-proof volumetric flasks. Aliquots of standard solutions were transferred with pre-cleaned micro-pipettes. Standards of known concentrations of OTC were prepared in duplicate from aliquots of standard solution. Samples were made to a total volume of 15 ml with 5% TCA, added to 50-ml glass centrifuge tubes, and processed like fry samples. Prior to use, all glassware was soaked in 10% HCL overnight and thoroughly rinsed with deionized water.

### Measurement of Fluorescence

Fluorescence was measured on a Turner 430 spectrofluorometer at a 390 nm excitation and a 520 nm emission wavelength. Disposable borosilicate glass culture tubes were used as cuvettes. The disposable cuvettes provided more consistent readings than quartz cuvettes which, unlike the disposable tubes, had to be decontaminated after use.

### Fry Samples

Prior to analysis, the entire digestive tract was excised from all OTC-fed and control fry. This was done to insure that recently consumed OTC in the stomach region was not being measured as residualized OTC. Hatchery fry were fed OTC in the form of Pfizer TM-50 (25  $\mu\text{g}$  OTC/0.2 mg TM-50), which was mixed in with Oregon Moist Pellet mash (OMP) at a ratio of 4.5% (w/w).

### Extraction

Individual fry were homogenized in a Waring commercial blender with 10-ml of 5% TCA and added to 50-ml pyrex centrifuge tubes. The blender cell was then rinsed with 5 ml of 5% TCA which was added to the centrifuge tube for analysis.

The centrifuge tubes were placed on a shaker table, agitated for 1 hour in the dark, and centrifuged at 2000 rpm for 30 minutes. The aqueous supernatant was added to another 50-ml centrifuge tube, and 15 ml of an

ethylacetate solution (13 ml of ethylacetoacetate made to 1000 ml with ethylacetate) were added. Samples were again agitated on the shaker table (for 10 minutes) and centrifuged at 2000 rpm for 5 minutes.

The top (ethylacetate) layer was transferred into a 50-ml centrifuge tube, to which 0.5 ml of 0.5 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 15 ml of 7 N  $\text{NH}_4\text{OH}$  were added. Samples were shaken for 10 minutes and centrifuged, as before, for 5 minutes. Finally, 45 minutes after the addition of the  $\text{NH}_4\text{OH}$ , 3-ml aliquots were removed from the top (ethylacetate) layer and added to 15-mm x 75-mm borosilicate culture tubes for reading against triplicate reagent blanks. Sample transfer at this last stage of the method was performed with automatic pipettes, the tips of which were cleaned with ethylacetate.

## RESULTS AND DISCUSSION

### Activation and Fluorescence Spectra of OTC

The optimal excitation and emission spectra for OTC (maximal OTC values with minimal reagent background fluorescence) of 390 nm and 520 nm were found by measuring the fluorescence of standard solutions (in concentrations of 0.94 and 18.8  $\mu\text{g}$  /15 ml TCA) and reagent blanks at emission wavelengths of 500-540 nm (Figure 1). These optimal wavelengths are in close agreement to those reported in previous studies by Ibsen et al. (1963), and Argauer and Gilliam (1974), and were selected on the basis of minimum blank values combined with sensitivity to the OTC-calcium complex, as well as minimal oscillation between replicate readings.

### Standardization

A linear relationship ( $r^2 = 0.997$ ) was found to exist between OTC concentration and fluorescence intensity over a wide range of duplicated concentrations of OTC standard solutions (Figure 2). More importantly, similar concentrations of OTC standard were added to homogenized OTC-negative control fry. By comparing both treatments, we found that the recovery of OTC was the same for both chemically pure samples of standard solution and those containing biological material.

### Extraction Efficiency

Because of the low levels of OTC anticipated to be present in fry samples, a number of experiments were conducted to determine the overall extraction efficiency of the method. We examined whether OTC was being lost: (1) in the ethylacetate- $\text{NH}_4\text{OH}$  phasing; (2) in the extraction from TCA into ethylacetate; or (3) through the action of phosphate interference from the biological material (see Kohn, 1961).

### Ethylacetate-Ammonium Hydroxide Phasing

To calculate the extraction efficiency of the ethylacetateammonium hydroxide phasing, the natural fluorescence of the ammonium hydroxide phase was

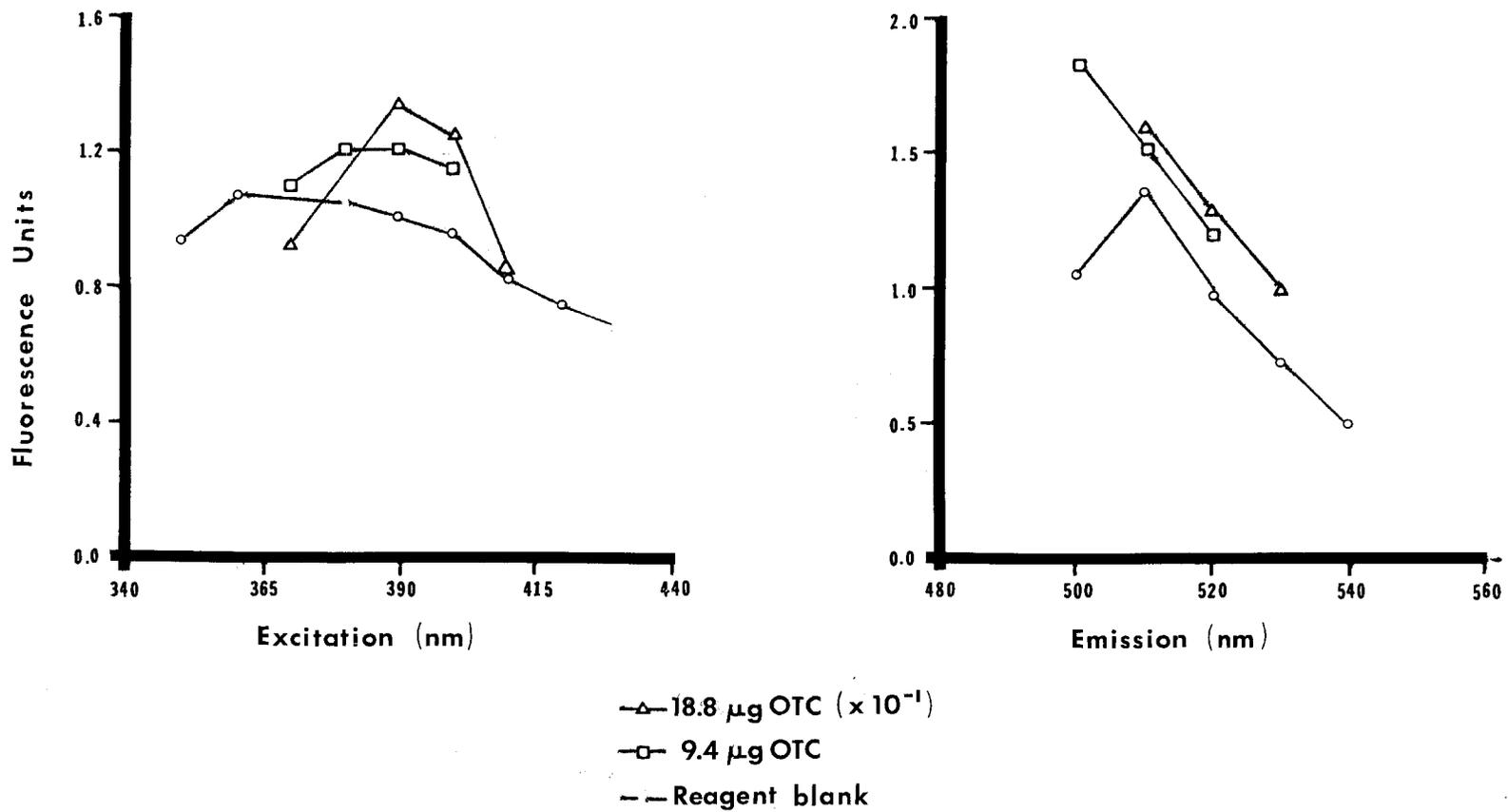


Figure 1. The relationship between excitation and emission wavelengths (nm) of the antibiotic oxytetracycline (OTC) indicating maximal excitation at 390 nm and optimal emission at 520 nm.

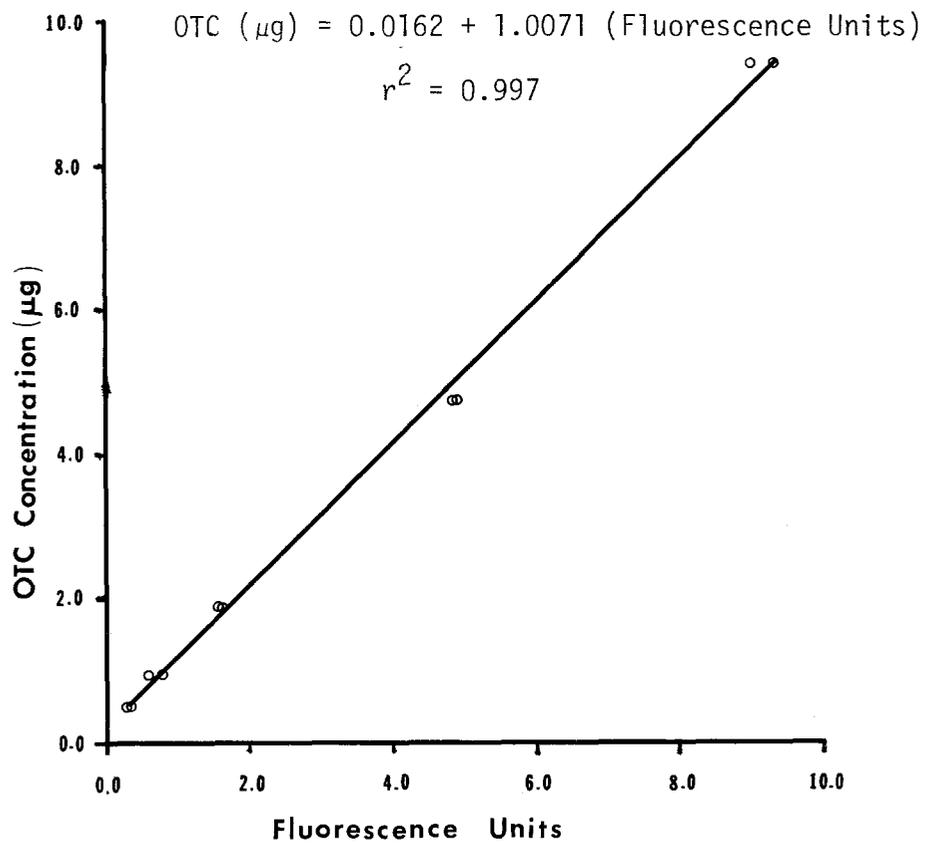


Figure 2. The linear relationship found between fluorescence intensity and the concentration of oxytetracycline (OTC).

determined from reagent blanks, and was subtracted from the fluorescence of the ammonium hydroxide phase in samples containing known amounts of OTC standard (ranging from 0.47 to 18.8  $\mu$ g). This yielded the concentration of OTC lost from ethylacetate into ammonium hydroxide. OTC recoveries were found to vary from 92-96% over the range of concentrations tested (Table 1). Thus, we concluded that the recovery of OTC was independent of the concentration present in the ethylacetate phase, and was virtually 100% efficient.

#### Extraction from TCA into Ethylacetate

To determine the extraction efficiency from TCA into ethylacetate, concentrations of standard solution ranging from 0.47 to 9.4  $\mu$ g were run through the entire method. The amount of OTC recovered was then compared to that recovered (after correction for extraction inefficiency) in ethylacetate shaken with the  $\text{NH}_4\text{OH}$  solution containing OTC standards. The extraction of OTC into ethylacetate was calculated to range from 32-42% for all concentrations tested, showing again that extraction of OTC by this method is relatively independent of the amount of antibiotic contained in the sample i.e., varying by a maximum of 10% (Table 2a).

We were concerned however, that the extraction efficiency tended to increase as the concentration of OTC increased. We therefore repeated the experiment, adding homogenized control fry to the same concentrations of OTC standard. The efficiencies calculated for homogenized control fry ranged from 30-35%, indicating that recoveries were not adversely affected by the presence of proteins, phosphates, or other biological materials (Table 2b). More importantly the extraction efficiencies were found to be independent of the concentration of OTC when biological material was being examined.

#### Effect of Reagents on OTC Extraction

In an effort to extract OTC more efficiently, different reagent preparations were used as suggested in the available literature. For example, it was found that the addition of calcium chloride, as reported by Kohn (1961) and Argauer and Gilliam (1974), resulted in higher extraction efficiencies than did magnesium chloride (Table 3) when using ethylacetate as an extractor. Furthermore, it was found, contrary to the data reported by Ibsen et al. (1963), that the addition of ethylenediaminetetraacetic acid (EDTA) to correct the initial fluorescence readings did not serve as a satisfactory internal blank.

We attempted to reduce organic contamination from the fry samples by filtering all samples through Whatman GF/D glass filters following homogenization and centrifugation. No increase in extraction was obtained using this method. Similarly, the addition of 1 ml of hexane (used to decrease the polarity of the alcohol phase) had no effect on the extraction of OTC from TCA into ethylacetate. Two further methods were employed in an effort to increase extraction efficiencies: samples of standard solutions were treated with 10% TCA instead of 5% TCA; and standard solutions were extracted into 5% and 10% TCA saturated with NaCl, as suggested by Ibsen et al. (1963). No additional recoveries were obtained using 10% TCA. In samples treated with TCA saturated with NaCl, extraction efficiencies could

Table 1. Recovery of oxytetracycline (OTC) from ethylacetate into the ammonium hydroxide phase. Recoveries were found to be independent of OTC concentration.

OTC concentration added ( $\mu\text{g}$ )	OTC concentration recovered ( $\mu\text{g}$ )	OTC recovered (% of total)
0.47	0.45	96
0.94	0.87	93
1.88	1.77	94
2.82	2.58	92
4.70	4.37	93
6.58	6.05	92
9.40	8.84	94
18.80	17.39	93

Table 2. The extraction efficiency of (A) oxytetracycline (OTC) from 5% trichloroacetic acid (TCA) into ethylacetate from samples containing OTC standard solution compared to the extraction efficiency of (B) OTC from 5% TCA into ethylacetate from samples of OTC standard containing homogenized control fry.

OTC concentration added ( $\mu\text{g}$ )	Reagent standards (A)		Homogenized fry standards (B)	
	OTC concentration recovered ( $\mu\text{g}$ )	OTC recovered (% of total)	OTC concentration recovered ( $\mu\text{g}$ )	OTC recovered (% of total)
0.47	0.15	32	0.16	35
0.94	0.32	34	0.31	32
1.88	0.63	34	0.56	30
4.70	1.97	42	1.58	34
9.40	3.72	40	2.95	31

Table 3. Comparison of fluorescence readings of reagent blanks, oxytetracycline (OTC) standard solutions, and control fry (OTC-negative) using different extractants.

Sample	Mean fluorescence			
	CaCl (0.5M <sup>2</sup> )	MgCl (0.5M <sup>2</sup> )	NH <sub>4</sub> OH (7 N)	NH <sub>4</sub> OH + NaCl (1.88N)
Reagent blank	28	33	30	35
OTC standard				
2.35 $\mu$ g	82	28	81	76
0.94 $\mu$ g	--	--	49	73
Control fry	23	21	41	60

be boosted by 10-15%, but the considerably greater variation in resultant fluorescence values rendered this method undesirable.

Although Argauer and Gilliam (1974) suggest the use of a dilute solution of ammonium hydroxide (59 g of NaCl + 125 ml concentrated  $\text{NH}_4\text{OH}$  diluted to 1 liter), we found that the use of 7N  $\text{NH}_4\text{OH}$  [as recommended by Ibsen et al. (1962)] resulted in lower reagent blanks, lower biological background fluorescence, and increased extraction of OTC (Table 3). As a result, we found an increased linearity over the range of concentrations tested, and an increase in reliability when assaying sub-microgram quantities of OTC. Thus, although our extraction efficiencies remained low (30-35%), they were not substantially lower than those reported by Argauer and Gilliam, (1974) (30%), Ibsen et al. (1963) (50%), and Kohn (1961) (50%).

#### Detection Limits

Ibsen et al. (1963) report 0.09  $\mu\text{g}$  OTC/ml as the minimal concentration they could detect with a variation of  $\pm 10\%$ , while Argauer and Gilliam (1974) report a detectable level of 0.16  $\mu\text{g}/\text{ml}$ . Even though the minimal concentration that we could detect in samples containing biological material (0.10  $\mu\text{g}/\text{ml} \pm 10\%$ ) agreed quite closely with their results, we did not find this to be a suitable operational definition, particularly as a criterion for making quantitative distinctions between OTC positive and negative fry.

Empirically, we found that the method could determine 0.47  $\mu\text{g}$  OTC/15 ml sample,  $\pm 0.14$   $\mu\text{g}$  (or 0.031  $\mu\text{g}/\text{ml} \pm 0.009$   $\mu\text{g}$ ) both in standard solutions, and in standard solutions added to known OTC-negative control fry. At this concentration, fluorescence values were 134% higher than the reagent background fluorescence. This value, however, cannot truly be reported as a detection limit, because it only represents the lowest concentration of OTC standard that was analyzed.

Using our regression equations correlating fluorescence with OTC concentrations, we computed a 95% prediction interval for our chemical blank i.e., 0.0  $\mu\text{g}$  OTC. We then defined the maximal positive variation (upper limit of the prediction interval) of the blank (0.012  $\mu\text{g}/\text{ml}$ , or 0.17  $\mu\text{g}/\text{sample}$ ) as the critical level above which a sample can be called OTC-positive. This value is somewhat lower than the limit of detection ( $C_L$ ) defined by the International Union of Pure and Applied Chemistry or IUPAC ( $C_L(K=3) = 0.023$   $\mu\text{g}$  OTC/ml) (see Winefordner and Long, 1983). However, we feel that the use of the 99.86% level of confidence recommended by IUPAC demands a precision not readily attainable given the biological nature of our investigations. Thus, we defined 0.17  $\mu\text{g}$  OTC/15-ml sample as being the minimally detectable concentration, above which samples were termed OTC-positive.

#### Recovery of OTC from Sockeye Salmon Fry

We tested the ability of the method to detect the presence of OTC in hatchery fry by feeding them OTC-medicated food (Figure 3). We found that as days of feeding increased the level of OTC/fry increased, as did the percentage of "marked" fish. By comparing reagent blank values with values

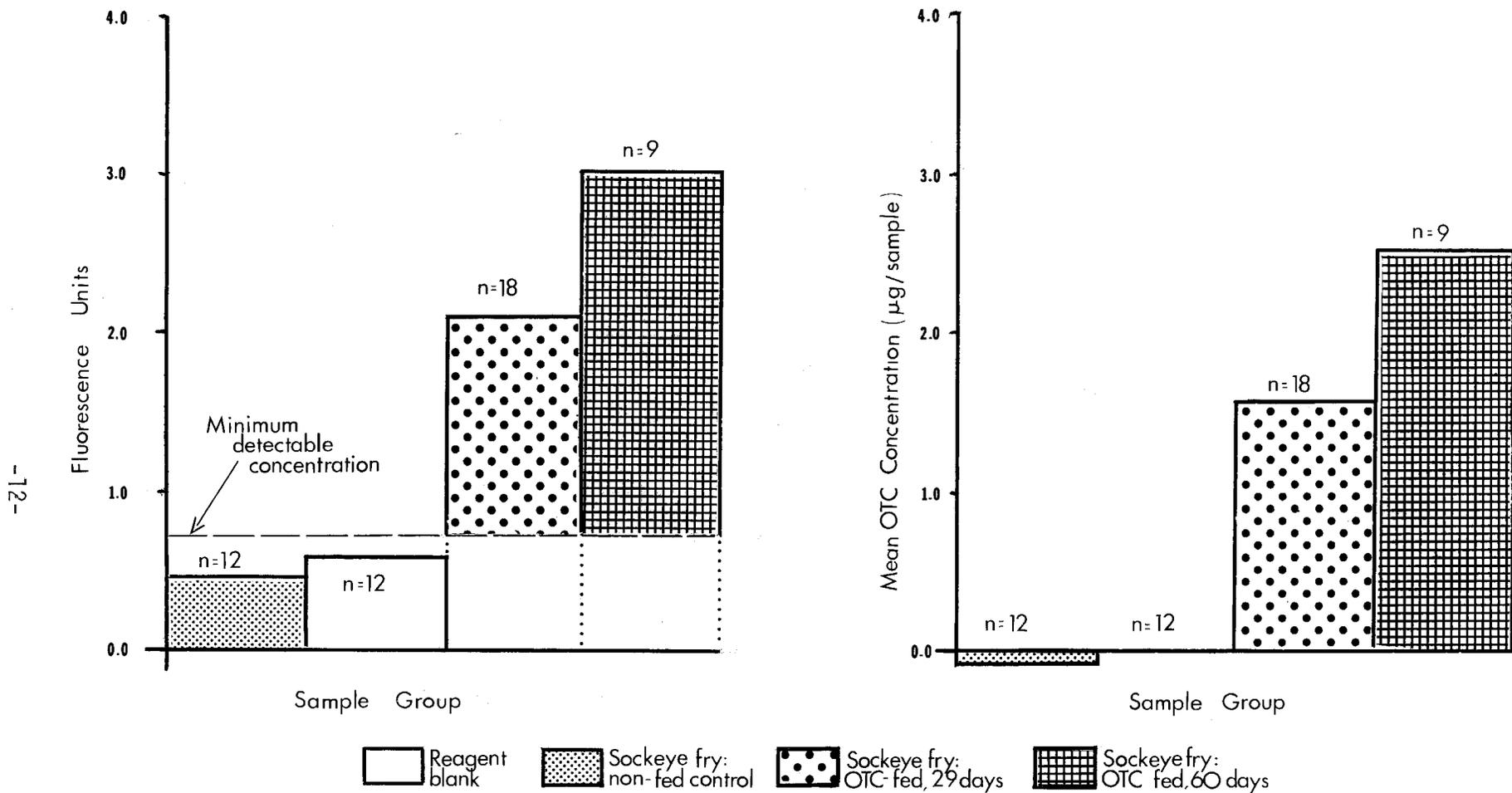


Figure 3. Comparison of fluorescence readings and quantity ( $\mu\text{g}$ ) of oxytetracycline (OTC) recovered from reagent blanks, non-fed control fry, and treated groups of sockeye fry fed OTC-medicated food (TM-50) for 29 days and 60 consecutive days. Also shown is the minimal detectable concentration of OTC ( $0.17 \mu\text{g OTC/sample}$ ), above which samples are termed OTC-positive.

from control, or known OTC-negative fry, we found all control fry to be below the minimally detectable concentration of OTC, and were thus concluded to be OTC-negative. Indeed, the fact that control fry, on the average, demonstrated a level of fluorescence below that of the reagent blanks (Figure 3), and showed a reduced extraction efficiency (Table 3), may be indicative of the presence of a chemical fluorescence inhibitor in salmonid fry. However, since the difference between control fry and reagent blanks was not statistically significant ( $p < 0.95$ ), this observation needs further testing.

After 29 days of OTC feeding, all test-fry (average weight = 0.4 g) were found to be OTC-positive, containing an average of 1.56  $\mu\text{g}$  OTC/fry, a level well above our minimally detectable concentration of 0.17  $\mu\text{g}$  OTC/fry. After 60 days of OTC-feeding, all fry (average weight = 1.0 g) were again found to be OTC-positive containing an average of 2.49  $\mu\text{g}$  OTC/fry. Again, in both test groups OTC-feeding produced "marked" fry readily distinguishable from known negative control fry, and increased feeding with OTC resulted in detectable increases in residualized OTC. Thus, we were able to generate a recoverable, internal OTC "mark" in sockeye fry which was used to quantitatively differentiate between treated and untreated groups of fish.

#### CONCLUSIONS

A method is presented to chemically extract and fluorometrically measure microgram and sub-microgram amounts of oxytetracycline (OTC) in sockeye salmon fry (*O. nerka*).

Standard curves demonstrate the reproducible linear relationship between OTC concentration and fluorescence intensity. In addition, the extraction of OTC did not vary in chemically pure samples of OTC standard solution when compared to samples containing biological material. However, at times, individual regression lines fell outside the 95% confidence interval computed for the OTC-fluorescence regression. Thus, we feel that OTC standards should be analyzed with each set of determinations.

Within the overall method, we found the phasing from ethylacetate into ammonium hydroxide to be 92-96% efficient, while the extraction efficiency of OTC from 5% TCA into ethylacetate was only 30-40% efficient. However, as the extraction efficiency of OTC was found to be independent of OTC concentration, recoveries of individual concentrations need not be adjusted for differential efficiencies.

We were able to determine the presence of OTC in fry samples at concentrations as low as 0.17  $\mu\text{g}$ /sample (0.012  $\mu\text{g}$  OTC/ml TCA). In addition, through trial feeding experiments, we followed the uptake of the antibiotic in OTC-fed fry, while at the same time we observed a relatively constant fluorescence from known negative control fry. Furthermore, our two treatment groups of fry that were fed for 29 days and 60 days showed an increase in OTC levels from 1.56  $\mu\text{g}$  OTC to 2.49  $\mu\text{g}$  OTC. This increased loading of OTC was consistent with the increased feeding time. Finally, fry samples

taken after 29 days and 60 days of feeding showed all fed fry to be positive (marked) while all control fry were found to be negative (unmarked). Thus, we feel the ability to quantify the levels of OTC present in fry can lead to the use of OTC as a non-toxic, internal tag enabling us to differentiate between "marked" and "unmarked" fry. With this procedure large numbers of sockeye fry can not only be inexpensively "marked" but also quantitatively identified; allowing us to compare mortality rates (in lake) between hatchery and wild fry. In addition, this method will afford us the opportunity to investigate mortalities found to occur in fry marked by fin-clipping, as well as errors in recognizing externally marked fish caused by both fin regeneration and natural fin loss.

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