IDEXX
Literature Cover Sheet

IDEXX #: 6A

Title: Evaluation of Autoanalysis Colilert in WasteWater

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Topic: Colilert use in wastewater

Highlights:

- Colilert can be substituted for Multiple Tube Fermentaion coliform analysis in wastewater with a 95% confidence limit.
- After isolation and identification of positive results the study showed Colilert was able to identify and quantify *E.coli* in wastewater and appears neither to be impaired by high heterotrophic bacteria counts, nor affected by the complex chemical matrix of the wastewater.

EVALUATION OF AUTOANALYSIS COLILERT[R] IN WASTEWATER

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ABSTRACT

A newly developed product for the simultaneous enumeration of total coliform bacteria and Escherichia coli. Autoanalysis COLILERT (AC), has been tested and approved for use with drinking water by the EPA. Applicability of the system to wastewater analysis has not been assessed. This study was designed to evaluate Colilert in a wastewater and marine receiving water matrix, and to determine its reliability as a substitute for the more labor intensive multiple tube fermentation procedure (MTF).

As part of the evaluation, a statistical analysis was made between the total coliform results obtained by both the MTF and AC procedures to determine comparability. Data is presented on the reliability of the AC method to accurately identify E. coli by comparing the results with those obtained by the API Enteric 20 System for coliform identification. Advantages and disadvantages of using the Colilert method over the multiple tube fermentation method are discussed.

INTRODUCTION

The relationship between fecal contamination of drinking water and disease has been known for centuries. Only since the late 1800's, however, have coliform bacteria been used as indicators of the disease potential of water, and only within the last seventy-five years have nov common statistical methods been used to determine the extent of contamination (1,2).

Two coliform detection and enumeration procedures are identified in Standard Methods for the Examination of Water and Wastevater (3): the multiple tube fermentation technique (MTF) which yields the most probable number (MPN) of coliform bacteria; and the membrane filter technique in which coliform colony forming units (CFUs) are counted directly.

Both methods have been used for years by the water and vastevater industries to monitor water quality for public health purposes. Each method has its ovn unique limitations. The membrane method yields results within tventy-four hours but its utility is significantly reduced as sample turbidity increases. The multiple tube method is less restricted by turbidity, but requires up to ninety-six hours to produce results.

In 1987-88 a new analytical system, Autoanalysis COLILERT^{R} was introduced (4) and reviewed (5,6,7). Colilert purported to be better and more specific than the standard methods, since it can yield quantitative data on both total coliform bacteria and Escherichia coli in 24 hours. The system was developed and has been used for drinking water analysis (8,9) and has received tentative approval by the EPA (10), but East Bay Municipal Utility District was interested in possible applications to wastewater and marine receiving water.

The purpose of this paper is to report on Colilert's performance compared to the existing methods, to verify the feasibility of using Colilert in treated wastewater and marine receiving water, to determine the reliability of the medium under a variety of test conditions, to identify potential problems using Colilert over conventional coliform methods, and to evaluate the cost effectiveness of converting partially or totally to this medium.

MATERIALS AND METHODS

Sampling- Samples were collected at irregular intervals from July, 1988 through January, 1989 from a total of seventeen locations (Table 1). The wastewater and receiving water sample stations are existing sites historically used for operations and regulatory coliform monitoring of the treatment process and receiving waters.

TABLE 1. Identification of the grab sample locations and characteristics of the water source.

Station Code	Water Source	Description
THRX	Wastewater	Treated wastewater; primary effluent
RCLX	Wastewater	Treated wastewater; reclaim water (secondary effluent after chlorination)
FEX02	Wastewater	Treated wastewater; chlorinated final effluent
BAYX01-17	SF Bay Water	Marine; receiving water
MISC	Untreated Wastewater	Wastewater and marine; marine intrusion to wastewater collection system

The primary effluent sample (THRX) was selected as a known positive control for total coliforms. No prior information was available on E. coli or fecal coliforms, however.

The secondary effluent sample (RCLX) was selected to identify coliform regrowth in water that was to be reused. EBMUD has encouraged non-potable reuse of treated wastewater for industrial uses; irrigation of recreation areas, public golf courses, and roadside ornamental vegetation; and for soil compaction. The reclaimed water is stored in a 100,000 gallon basin and is available at no charge to users.

The final effluent sample (FEX02) is the District's NPDES compliance monitoring station. Under normal operating conditions coliforms are not detected, therefore, this station represents a negative control and was included as the most sensitive wastewater monitoring point.

The BAYX01-17 sampling locations are marine water stations in San Francisco Bay established as NPDES compliance monitoring points for receiving water.

A few additional samples were collected and identified as MISC. These samples were part of a short term side study involving saltwater intrusion into the District's interceptor collection system.

All samples were collected in 250 mL ground-glass, mushroom-stoppered Wheaton bottles or 500 mL Pyrex®, bottles with high temperature rings and caps. The bottles contained sodium thiosulfate to reduce any residual chlorine and were cleaned and sterilized using procedures detailed in Standard Methods. Samples were either processed within one hour of collection or stored at 4° C while in transit then processed immediately upon receipt at the Laboratory (always within 6 hours).

Colilert Method (AC) - Autoanalysis Colilert is packaged in sterile 13 x 100mm culture tubes with sufficient dehydrated medium for a 10 mL sample inoculum per tube (11). Generally, samples for Colilert analysis were set-up as 4 or 6-row dilutions with each row containing five tubes and each dilution row, one-tenth the concentration of the preceding row. All dilutions were made with sterile deionized dilution water (SDDW), and were inoculated directly into the Colilert culture tubes.

The culture tubes were incubated at 35 ± 0.5° C for 24 hours then each tube was examined for yellow color production indicating a positive total coliform result. Positive total coliform tubes were reexamined for fluorescence using a 366 nm, longwave UV hand lamp. Strongly fluorescing tubes were assumed to be positive for E. coli. Most Probable Number (MPN) values per 100 mL of sample were calculated for total coliform and E. coli using standard MPN tables (3).

Analytical and Confirmation Process - AC, MTF, and heterotrophic plate count (HPC) tests were simultaneously set-up using the same sample. MPNs were inoculated into 4- or 6-rows each containing 5 tubes. The sample was processed and total coliform MPN values were obtained for both the AC and MTF methods. Fecal coliform, E. coli and heterotrophic bacterial counts also were recorded. As the study progressed, it was determined that heterotrophic bacteria had no apparent interfering effect on analytical results and the HPC analysis was discontinued. E. coli positive Colilert tubes were isolated, reconfirmed in Colilert and identified using the API 20E System®.

RESULTS

Colilert vs. MTF Total Coliform Analysis - A total of 95 treated wastewater and 34 marine receiving water samples were split and analyzed for total coliforms by both the Colilert and MTF methods. Of the treated wastewater samples taken,

67 (70%) of the samples produced MPN values > 2 per 100 mL by both procedures. For the marine samples, 29 samples (85%) had detectable total coliform levels in both the Colilert and MTF tests.

The first data review consisted of a direct comparison of the paired MTF and AC MPN results. If the AC MPN value fell within the 95% confidence limit range of the MTF value, the pair was recorded as being equivalent, otherwise it was recorded as being either greater than or less than the MTF value. The results of this comparison are summarized by station in Table 2.

Table 2. Comparison of Colilert and MTF total coliform MPN results for all wastewater and marine samples with 95% confidence limits applied.

	Treated Wastewater and Marine Stations			
MPN Result Comparison	FEXO2 (31)[1]	RCLX (32)	THRX (32)	BAYX (33)
AC < MTF	0% (0)	9% (3)	9% (3)	9% (3)
AC = MTF	100% (31)	44% (14)	81% (26)	64% (21)
AC > MTF	0% (0)	47% (15)	9% (3)	27% (9)

[1] Values in parentheses represent the number of samples in that category.

cause of the significant number of samples which produced MPN values of < 2 in one or both of the paired results (30% in the wastewater samples and 15% in the marine samples), a second comparison was made excluding these data. By eliminating these results from the comparison the totals were lower, but the overall relationships remained the same. The disproportionate number of AC results from the RCLX and BAYX stations which produced MPN values higher than their paired MTF values at the 95% confidence level could not be explained without more extensive investigation.

The total coliform MPN data were also examined by regression analysis. For this analysis, paired samples producing all negative tubes by one or both of the analytical methods were excluded. Two regression analyses were made, the first on the combined treated wastewater data from the FEXO2, RCLX and THRX stations, and the second on the marine receiving water data from the BAYX stations. Comparison of the two methods for wastewater samples produced an r-value of 0.886 and an r^2-value of 0.785 (Figure 1). It is noteworthy that the regression analysis was performed on MPN values which are statistically produced numbers with broad confidence limits. The significance of such a strong correlation given this consideration can not be overlooked.

A similar regression analysis was made on the BAYX data which produced much less significant results. For marine receiving waters the r-value was 0.706 and the r^2-value was 0.498 (Figure 2). The poor correlation was emphasized

when the two highest data pairs were dropped and the regression analysis was repeated. The recalculated r-value was 0.591 with an r^2-value of 0.349.

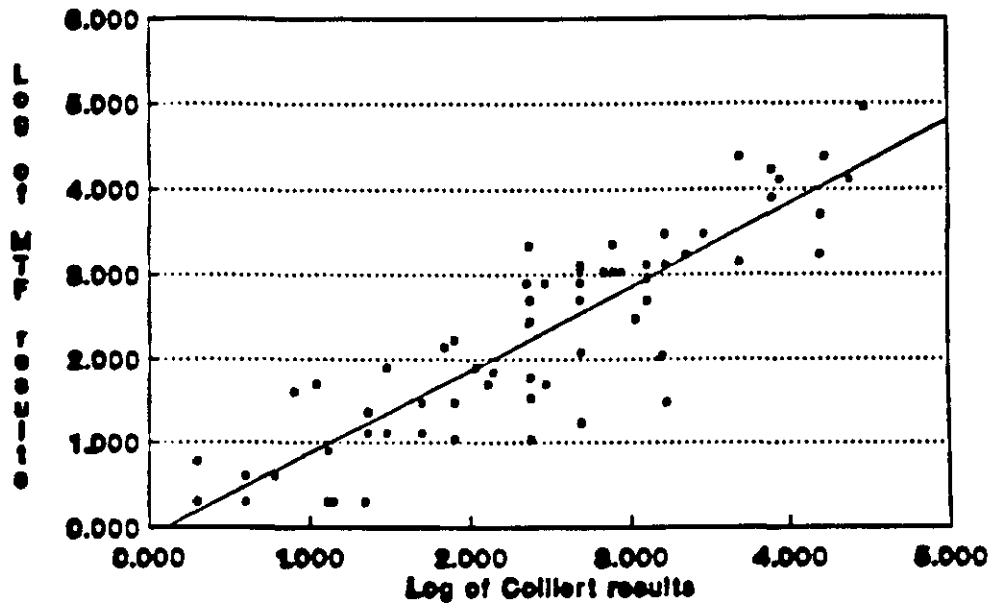

FIGURE 1. Illustrated is the $\log_{10}$ transformed data from the wastewater MPN values produced by AC and MTF and compared by regression analysis where y = 0.981x - 0.112, with r = 0.886.

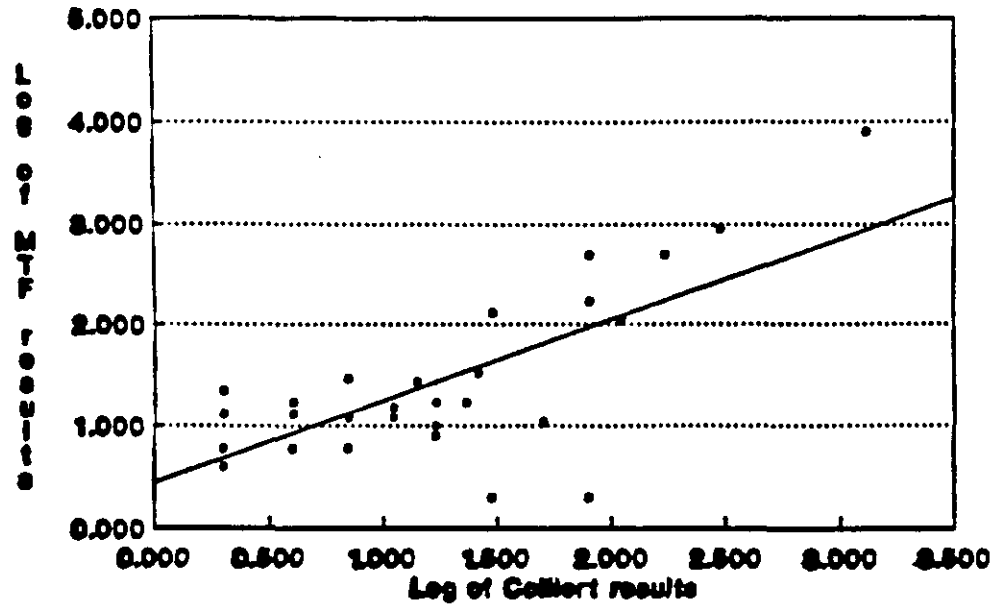

FIGURE 2. Illustrated is the $\log_{10}$ transformed data from the marine receiving water MPN values produced by AC and MTF and compared by regression analysis where y = 0.619x + 0.348, with r = 0.706.

Colilert vs. Fecal Coliform Analysis - As part of the evaluation process, fecal coliform (FC) MPN values were determined following the MTF analysis. It was assumed that the Colilert E. coli MPN values should never be significantly greater than the fecal coliform MPN values since the former is a subset of the latter. In all but one of the 127 marine and wastewater paired data sets, the fecal coliform MPN was greater than or equal to the E. coli value (Table 3).

Table 3. Comparison of Colilert E. coli and MTF fecal coliform MPN results for all wastewater and marine samples with 95% confidence limits applied.

	Treated Wastewater and Marine Stations			
MPN Result Comparison	FEX02 (30)[1]	RCLX (32)	THRX (32)	BAYX (33)
AC < FC	0% (0)	0% (0)	50% (16)	21% (7)
AC = FC	100% (30)	97% (31)	50% (16)	79% (26)
AC > FC	0% (0)	3% (1)	0% (0)	0% (0)

[1] Values in parentheses represent the number of samples in that category.

Coliform Identifications - From selected AC and FC tubes positive for E. coli and fecal coliforms, additional testing was performed to verify the presence of E. coli. In AC positive tubes the following isolation steps were used:

- A loop of the suspension is streaked onto either EMB, BHI or MacConkey Agars and incubated for 24 to 48 hours at 35° C.
- Selected isolates are transferred to lauryl tryptose broth (LTB) and a rehydrated tube of Colilert (confirmation of original result).
- After 24 hours incubation, a loop of the LTB suspension is streaked a second time onto one of the agars and incubated 24 to 48 hours at 35° C.
- Isolated colonies are transferred to a nutrient agar (NA) slant and saved for API identification.
- Concurrent with preparing fresh NA slants for APIs, the culture is confirmed a second time in Colilert.
- The pure culture is identified by API analysis.

The FC positive tube identifications followed a similar double isolation procedure, in lieu of Colilert, however, LTB medium is substituted for the confirmation step.

In wastewater and marine receiving water samples a diverse population of coliforms was anticipated, therefore, it was expected that the presence of E. coli could not be confirmed in every case. A total of 236 identifications were made from over 300 isolations using the API Enteric 20 System® (Table 4). From those AC tubes which fluoresced and were considered E. coli positive, the most frequently identified organism (35% of the identifications) was indeed E. coli.

As expected, there was a lower percentage of E. coli identifications in the FC tubes which related to the reduced selectivity of medium used. As a rule, however, those organisms which predominated in AC also predominated in the FC tubes. AC tubes which produced questionable or ambiguous E. coli results were tracked separately.

Table 4. Species identification and distribution of coliform bacteria found in marine and wastewater, and cultured in AC and FC.

	% Colilert Isolates		% MTF Isolates	Distribution	
Species ID	AC+[a]	AC?[b]	FC+[c]	WW[d]	RW[e]
Citrobacter freundii	16	20	16	24	4
Enterobacter aerogenes	1	7	2	3	2
E. agglomerans	1	7	5	3	4
E. cloacae	5	15	19	14	7
E. sakazakii	0	0	1	1	0
Escherichia coli	35	13	14	21	25
Hafnia alvei	0	1	0	0	1
Klebsiella oxytoca	0	6	5	4	1
K. ozaenae	0	1	0	1	0
K. pneumoniae	33	20	34	24	40
Morganella morganii	0	0	2	1	0
Proteus vulgaris	0	0	2	0	1
Serratia liquefaciens	3	3	2	3	1
S. marcescens	0	3	0	1	0
Vibrio alginolyticus	2	1	0	0	4
V. fluvialis	5	3	0	0	9

[a] A total of 101 identifications from E. coli positive Colilert tubes.
[b] A total of 71 identifications from E. coli questionable Colilert tubes.
[c] A total of 64 identifications from fecal coliform positive tubes.
[d] A total of 155 identifications from treated wastewater samples.
[e] A total of 81 identifications from marine receiving water samples.

At the conclusion of the study, a new saltwater Colilert formulation was evaluated in parallel with the original Colilert formulation and the MTF procedure. The results of this limited comparison study are reported in Table 5. The new formulation was prepared in response to a number of problems the District was having with false or questionable positive E. coli results in the receiving water samples. In many of the culture tubes there was no distinct fluorescence. Instead, there were AC tubes which were unquestionably positive, those which were unquestionably negative, and a large group which seemed to fall some place between the two extremes. The problem with the original formulation was managed by extending the incubation time an additional 2 hours and recording all questionable tubes as negative.

Table 5. Comparison of MTF fecal coliform and E. coli MPN results for marine receiving waters using two different formulations of Colilert.

Station	FC MPN	AC_{old} MPN	AC_{new} MPN
BAYX01	80	< 2	23
BAYX05	17	2	11
BAYX06	30	< 2	8
$BAYX06_{dup}$	22	< 2	13
BAYX09	17	< 2	2
BAYX13	8	< 2	4
BAYX17	110	< 2	110

DISCUSSION

Autoanalysis Colilert is a product which uses enzymes unique to coliforms to cleave the bonds between an indicator and nutrient component of two organic compounds. The indicator/nutrients ONPG (ortho-nitrophenyl-B-d-galacto-pyranoside) and MUG (4-methyl-umbelliferyl-B-d-glucuronide) respectively identify and enumerate total coliforms and E. coli. For ONPG the nutrient portion (galactopyranoside) is metabolized, and the indicator portion (ortho-nitrophenyl) is released. Separation of the indicator results in a visible change from a colorless liquid to a yellow liquid (12). Similar enzymatic actions take place when MUG is split into its nutrient portion (glucuronide) and the indicator portion (methylumbelliferone) by an enzyme (glucuronidase) specific to E. coli. When methylumbelliferone is exposed to UV light at 366 nm it fluoresces.

The results of this investigation confirm that Colilert can be substituted for the MTF total coliform analysis in wastewater. MPN values for the AC and MTF methods were equivalent at the 95% confidence level in 75% of the samples (Table 2). When the wastewater data are combined and subjected to regression analysis, an r-value of 0.886 and a regression line slope of approximately one ($y = 0.981x - 0.112$) supports comparablity (Figure 1).

The marine receiving water results are not as clear. Table 2 shows that there is a tendency for the AC analysis to produce higher total coliform MPN values than those produced by the MTF method. This is somewhat supported by the slope of the regression line ($y = 0.619 + 0.348$), though the correlation is not good. Two opposing observations may be made from these data, either Colilert does a better job of resuscitating coliforms stressed by exposure to highly saline bay water than does the MTF method, or some form of color interference is producing a significant number of false total coliform positives in Colilert. Our color and turbidity data on the marine samples, the reconfirmation data with Colilert, and the work of Edberg, et al (11) on fresh water samples suggest that coliforms are able to metabolize ONPG producing a reduced color intensity even

though stressed by high osmotic pressures which could retard or inhibit growth and/or fermentation in conventional media.

Many investigators have attempted to use the specific biochemical response of MUG in a number of media to enumerate E. coli (13,14) under a variety of conditions including drinking water (8,10,11), shellfish assays (15), sewage and surface waters (16), but little has been done with MUG in marine waters (17). Much of this study concentrated on the detection of E. coli in wastewater and marine water.

From the beginning, all attempts to make clear distinctions between positive and negative MUG responses for E. coli in marine water samples were met with frustration. The cause of the interference appeared not to be due to any form of biochemical activity, but simply a reflection of light from an inorganic suspension in the culture tube. Extraordinary efforts were made at circumventing this problem. The first attempted solution was to use a saltwater Colilert blank in place of the standard provided with the Colilert medium. The rational was that if there were some form of background interference, it could be taken into consideration when interpreting the result. This worked reasonably well, and became the standard procedure in subsequent analyses.

Several attempts followed at improving the procedure by reducing or removing the interfering turbidity. The next effort was to filter the sample through a 0.45 µm membrane filter and transfer the filter to a rehydrated Colilert tube. This proved to be so cumbersome that it was quickly rejected as an alternative method.

Colilert E. coli positive and E. coli questionable suspensions were then subjected to high speed centrifugation followed by UV spectrophotometric analysis at an excitation wavelength 366 nm and an emission wavelength of 470 nm. Emission peak heights were initially recorded, the suspension was centrifuged and the supernatant was reanalyzed. The before and after centrifugation results indicated that there was no substantial shift in peak heights. For E. coli positives deflection ranged from 26 to 70% and for E. coli questionable suspensions from 1 to 30%. Though this work proved to be academically interesting, it was neither a practical improvement in the method nor a useful tool for properly identifying questionable positives.

The District presented our problems with questionable E. coli positives to the product manufacturer. After much deliberation, it was theorized that the most likely cause was an inorganic reaction between the phosphate buffers in Colilert and the dissolved salts in seawater forming an insoluble precipitate. It was also suggested that either the precipitate was fluorescing itself, or was scattering the UV light in such a way as to mimic fluorescence.

The proposed solution was to reformulate the product using an organic rather than the original inorganic buffer. In December 1988, the District received a limited supply of the new Colilert product, and parallel tests were run using the MTF procedure, the original AC formulation (AC_{old}) and the reformulated AC (AC_{new}). The FC and E. coli MPN results for both formulations of this side-by-side study are reported in Table 5.

Though the number of samples is small, it is clear that the MPN values recorded for E. coli with the new formulation are considerably higher than those for the original formulation, and are more in line with the observed FC MPN values. The low numbers for the original formulation are most certainly a result of strict adherence to the previously established policy of all questionable results being recorded as E. coli negative. Curiously, the total coliform MPN results of the new formulation were greater than or equal to both the MTF and the AC_{old} in six of the seven samples. Whether this was real or simply coincidence was not validated.

Summarizing, the AC procedure does provide data comparable to conventional standard methods for total coliform enumeration in treated wastewater. The results of isolate identification analyses indicate that Colilert is able to identify and quantify E. coli in wastewater and appears neither to be impaired by high heterotrophic bacterial counts, nor affected by the complex chemical matrix of the District's wastewater. In marine water, the data indicate that the original Colilert formulation is unable to produce results which correlate well with currently used methods and that difficulties in interpreting E. coli results minimize this formulation's usefulness. Preliminary testing on a new, organically buffered Colilert formulation, however, suggest that problems encountered with the original formulation may have been solved, and that early results certainly warrant further investigation. Finally, a cost analysis comparing AC to the MTF method for total and fecal coliforms in wastewater and receiving waters indicates that Colilert is less costly when considering all associated material and labor costs.

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