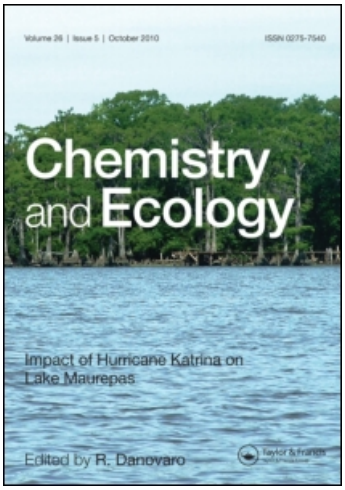

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

Combined Toxicity of Ethylamine and Methylamine in an Aquatic Bioassay System Using the Marine Bacterium *Vibrio Harveyi*

K. W. Thomulka[a]; J. H. Lange[b]
[a] Philadelphia College of Pharmacy and Science, Philadelphia, PA, USA [b] Envirosafe Training and Consultants, Pittsburgh, PA, USA

To cite this Article Thomulka, K. W. and Lange, J. H.(1995) 'Combined Toxicity of Ethylamine and Methylamine in an Aquatic Bioassay System Using the Marine Bacterium *Vibrio Harveyi*', Chemistry and Ecology, 11: 4, 183 — 192
To link to this Article: DOI: 10.1080/02757549508039069
URL: http://dx.doi.org/10.1080/02757549508039069

Chemistry and Ecology, 1995, Vol. 11, pp. 183–192
Reprints available directly from the publisher
Photocopying permitted by license only

COMBINED TOXICITY OF ETHYLAMINE AND METHYLAMINE IN AN AQUATIC BIOASSAY SYSTEM USING THE MARINE BACTERIUM *VIBRIO HARVEYI*

K. W. THOMULKA[1] and J. H. LANGE[2]

[1]*Philadelphia College of Pharmacy and Science,*
600 South Forty-Third Street, Philadelphia, PA 19104 USA
[2]*Envirosafe Training and Consultants,*
P.O. Box 114022, Pittsburgh, PA 15237 USA

(Received 2 February 1994; in final form 5 April 1995)

A combined or mixture toxicity study for two organic chemicals, ethylamine and methylamine, was performed using a bioluminescence-reduction bioassay. This assay employs the marine bacterium *Vibrio harveyi* as the test organism. An additive index method was used to evaluate the combined toxicity of these two chemicals at each of their estimated median effective concentrations (EC_{50}). Combinations or mixtures at 20% intervals of the EC_{50}, using both the isobole plot and the isobologram method, were invesitgated. Mixtures of ethylamine and methylamine exhibited an additive effect based on evaluation of confidence intervals using the isobole and isobologram plots. Combined concentrations of these chemicals using their EC_{50} values had a synergistic effect based on the additive index method. A statistical difference was observed between the most descriptive antagonistic and synergistic mixture intervals. No other intervals, including the comparison between single chemical evaluations, were statistically different.

KEY WORDS: Bacterial bioassay, chemical mixture studies, environmental toxicology, combined toxicity, ethylamine/methylamine.

INTRODUCTION

Most environmental toxicology studies evaluate the effects of a single chemical on an organism (Thomulka *et al.*, 1993a). In the real world, most chemical exposures occur as a result of mixtures, not individual toxicants (Kreitzer and Spann, 1973; de Zwart and Slooff, 1987; Deneer *et al.*, 1988; Dirilgen and Inel, 1994). Chemical combinations often occur as a result of two or more materials being applied as a mixture to increase their effectiveness, which can result in smaller application amounts and reduced costs for each of the chemicals applied (Marking, 1985). Because of the joint toxicity and the potential for pollutant releases from different sources or operations, combined or mixture toxicity can have dramatic effects on an ecosystem that would not normally be seen with a single toxic agent (Marking, 1985). The major drawback in studying multiple, joint or combined chemical toxicity is the complexity and difficulty in performing these evaluations (Konemann, 1981; Spliid and Torslov, 1994).

This investigation evaluated the combined or mixture toxicity of ethylamine (EA) and methylamine (MA) using a previously described bioassay procedure that employs

the marine bacterium *Vibrio harveyi* (Thomulka *et al.*, 1992, Thomulka *et al.*, 1993b). Ethylamine and methylamine are common organic solvents used in many commercial and industrial activities (Hathaway *et al.*, 1991). The common use of these chemicals makes it likely that they will be found jointly as contaminants in the aquatic environment. Methylamine, by both its oral toxicity (LD_{50} 0.02 mg/kg – for rats) and EC_{50} values in direct and growth bioassays using *Photobacterium phosphoreum* and *Vibrio harveyi*, is descriptively more toxic than ethylamine (LD_{50} 0.4 mg/kg – for rats) (Williams and Burson, 1985; Thomulka *et al.*, 1992). Combined or mixture toxicity was evaluated for synergistic/antagonistic/additive effects using an isobole plot procedure (Roales and Perlmutter, 1974a), isobologram method (Altenburger *et al.*, 1990) and an index formula method (Marking and Mauck, 1975). A statistical comparison was also performed for mixtures (Roales and Perlmutter, 1974a).

Isobole plots are graphic representations of the combined result of two chemicals that are administered in different ratios of their EC_{50} concentration. If the mixture of chemicals is additive, combined ratios will fall along a straight line between the individual EC_{50} values. Combination ratio values falling below or above this line are suggestive of being synergistic or antagonistic (Marking, 1985). Graphic representations of isobole plots have been used to categorize mixtures descriptively. Inclusion of confidence intervals (CI) for combination ratios and individual EC_{50} values have been used in statistical evaluation of these plots (Thomulka and Lange, 1995).

Isobolograms are the graphic representation of two chemicals mixed in various proportions of their individual EC_{50} concentrations. Resultant effects of the mixture concentration is multiplied by the outcome, as counts per second, for *V. harveyi* (Thomulka and Lange, 1994). This proportion is used in determining each point in the isobologram (Altenburger *et al.*,1990). An isobologram can be evaluated statistically in the same manner as the isobole plot.

Both the isobole plot and isobologram are based on the same concept and graphically present mixture data by representing ratio values of the chemicals. This two-dimensional representation can be used to conceptualize combined effects. Inclusion of statistical parameters strengthens the ability to interpret these plots.

MATERIALS AND METHODS

The bioassay used *Vibrio harveyi*, a marine bacterium, which was obtained from David Lopota at the Naval Oceans Systems Center, San Diego, CA. Stock cultures of this organism were maintained on nutrient agar containing 3% NaCl (NA) (Thomulka *et al.*, 1993a). An overnight nutrient broth culture (ONC) containing 3% NaCl with 25 ml of medium in a 125 ml Ehrlenmeyer flask was inoculated from a stock culture, capped loosely and incubated for 24 hours. Since oxygen is required for luminescence (counts of light) a Morton Closure was used to cap the flask. All incubations were at 23 °C (Thomulka *et al.*, 1993a; Thomulka and Lange, 1994). Imidazole buffered saline with sucrose and nutrient broth (NB) (IMS-SNB) was made by adding 10 ml of NB and 5 grams of sucrose to 2 M imidazole dissolved in 30% saline. The pH was adjusted to 7.0 with NaOH and the final volume adjusted to 1 litre with 30% saline. This was a 10x stock solution which was kept refrigerated. It was diluted 1:10 in distilled water before use.

A cell suspension was prepared by adjusting the absorbance of the ONC to 0.5 at 600 nm and then further diluted 1:10. Both the adjustment and dilution used cold IMS-SNB. The diluted ONC was continuously refrigerated or maintained in an ice bath until use. Time period for use of this diluted ONC did not exceed 2 hours.

Stocks of ethylamine (EA) and methylamine (MA) were prepared fresh for each experiment. All stock solutions were used within 2 hours of preparation. The control tube, which functioned as a blank, consisted of 950 µl of IMS-SNB and 50 µl of cell suspension. No agent was added to control tubes. Each reaction tube (polystyrene tube, 12 × 55 mm) contained 10–100 µl of agent (ethylamine and/or methylamine), 50 µl of cell suspension and sufficient IMS-SNB for a final volume of 1,000 µl. All tests were conducted using nominal concentrations for experimental toxicants.

The assay was initiated by adding, at 20 second intervals, 50 µl of cell suspension to each tube, vortexed and placed in a water bath at 23° (Ribo and Kaiser, 1987; Thomulka *et al.*, 1993a). The cell suspension was vortexed briefly after addition to every tenth reaction or control tube to ensure uniformity. Tubes were incubated with the chemical(s) for one hour and the luminescence (counts of light production by the bacterial-bioluminescence) was then determined. Bacteria are not growing in the direct assay, although the buffer contains sufficient nutrients for at least five hours of luminescence (Thomulka and Lange, 1994).

After incubation, the tubes were counted individually for 6 seconds to determine luminescence (counts). Values given in this paper are counts per second. As a tube was being counted, the next was removed from the water bath, dried with a paper towel, vortexed and placed in the photometer. One complete cycle (load, count, print and unload) required 20 seconds. The photometer used was a Los Alamos Diagnostic Model 735 Luminometer which provides a printout of counts (luminescence).

The estimated median effective concentration (EC_{50}) was determined singly for each of the organic chemicals, ethylamine and methylamine, using graphic methods (Dutka and Kwan, 1981; Thomulka *et al.*, 1993a). EC_{50} values are reported in ppm (Somasundaram *et al.*, 1990). Confidence intervals (CI) at 95% were determined for EC_{50} values using a method described by Frumin *et al.* (1992), modified for bacterial assays by Thomulka *et al.* (1993b). The numerical value for n was 20 and 34 for combination mixtures (isobologram and isobole plots) and additive index mixtures, respectively, used in determining the CI, and represents the number of replicates analyzed for each chemical (Thomulka *et al.*, 1993a; Thomulka *et al.*, 1993b; Thomulka *et al.*,1993c). Each replicate consisted of 4 experimental tubes analyzed at the same time. Replicates were analyzed as separate experiments and were performed at different time periods. CI intervals for luminescence counts were determined using standard statistical techniques (Daniel, 1991; Timko and Downie, 1992). All mixtures for the isobole and isobologram were performed with a given fraction of each chemical, MA and EA, that would arithmetically, using the additive equation model, equal 100% of the EC_{50}. For example, a 80%/20% of EA/MA combination represents 80% of EA's and 20% of MA's EC_{50} which for this combination mixture was 116 and 17.4 ppm, respectively.

Combined or mixture effects of the two organic chemicals (ethylamine and methylamine) together, were evaluated at the same time by three methods used in mixture studies, an index (additive index) (Marking and Mauck, 1975), an isobole plot procedure (Roales and Perlmutter, 1974a; Roales and Perlmutter, 1974b) and an isobologram plot method (Altenburger *et al.*, 1990).

Isobole plots were reported as counts for each solution composition. By graphing these data, the shape of the curve is used qualitatively in determining the type of effect. Toxicity was determined for each organic compound singly and in fractional combination (Roales and Perlmutter, 1974a; Marking, 1985). Combined effects employed 20% intervals from the EC_{50} concentration of each organic chemical as previously described (Roales and Perlmutter, 1974a; Roales and Perlmutter, 1974b). The intervals tested were 0.2 (20%), 0.4 (40%), 0.6 (60%), 0.8 (80%) and 1.0 (100%) times the EC_{50} for one chemical with the other chemical constituting the remaining percentage of the mixture, as determined by its EC_{50} times the fraction amount, to determine the combination ratio.

Thus, possible combinations are 20%/80%, 40%/60%, 60%/40%, 80%/20% for each chemical combination. The EC_{50} concentrations for ethylamine and methylamine are 145 ppm and 87 ppm, respectively (Thomulka *et al.*, 1992). Exposure concentrations are determined by multiplying the chemical's EC_{50} by contribution (i.e. 20% is 0.2). To quantify these values better, an overlap of their CI with the CI and the straight line between 100% concentrations of each individual chemical is considered additive toxicity (Altenburger *et al.*, 1990). Overlap values below and above these CI ranges are qualitatively representative of either synergism or antagonism (Marking, 1985; Altenburger *et al.*, 1990).

Isobolograms are based on the same concept as isobole plots except that this technique plots the two chemicals against each other in their individual proportions (Altenburger *et al.*, 1990). The mixture effect is determined from the calculated value and its CI for the individual chemical and combinations as described for the isobole plot. Thus, the resultant effects of the mixture can be determined quantitatively and qualitatively in the same manner as isobole plots (Altenburger *et al.*, 1990).

A concentration at the EC_{50} value for each chemical (ethylamine – EC_{50} = 87 ppm and methylamine – EC_{50} = 145 ppm) was added together, at the same time, to the reaction mixture and luminescence was determined as described. This combined value was then compared to luminescence for each chemical individually at its EC_{50} concentration to determine the additive index value (Marking and Mauck, 1975). For this additive index evaluation, the experimental reaction tube contained 145 and 87 ppm of methylamine and ethylamine, respectively.

The additive index(s) was calculated by summing the contributions of counts for combined EC_{50} chemical concentrations. These counts (luminescence) were used in the additive index equation, below, to determine the sum of toxic action as previously described (Marking and Mauck, 1975; Marking, 1985):

$$S = Am/Ai + Bm/Bi$$

where A = chemical A
B = chemical B
S = sum of toxic action
m = mixture of each at its EC_{50} value, as represented by counts
i = individual EC_{50} value, as represented by counts.

The range of effect for the additive mixture was determined by substituting the upper and lower CI for the average number of counts that corresponds to the EC_{50} value as previously described (Marking, 1985). Negative values of the index suggest qualitatively a less than additive toxicity, with zero being additive and positive values being

suggestive of synergistic effects (Marking and Mauck, 1975). To quantify this index better, the CI overlap with zero can be evaluated (Roales and Perlmutter, 1974a; Marking, 1985). If no overlap exists, the mixture is considered quantitatively to deviate from additivity.

Evaluation of combinations with each other was performed using the t-test as described by Roales and Perlmutter (1974a). A probability value was calculated for various combinations.

CIs and statistical comparisons were both represented and evaluated at the 95% level. Each of these methods employed the counts of bioluminescence in determining the mixture effects.

RESULTS AND DISCUSSION

Combined and single toxicity of ethylamine and methylamine at various combinations of 20% intervals of EC_{50} values are shown in Table I. Toxicity was determined by change in bioluminescence of the test organism, *V. harveyi*. A decrease in bioluminescence (counts of light), as compared to the control or comparison test, represents the toxicity of the chemical exposure of the organism. The larger this difference, the greater the toxicity. Counts can be related to the effectiveness of the chemical as represented by its median reduction in bioluminescence (EC_{50}) as previously described (Thomulka *et al.*, 1992; 1993a). Chemicals in this study were evaluated as mixture components at the same time (Dirilgen and Inel, 1994).

The graphic (isobole) representation (Figure 1) of these data shows variation among the different combinations tested. The 80% ethylamine/20% methylamine mixture supports a greater than additive (synergism) effect. However, the combinations 60%/40% and 40%/60% of ethylamine/methylamine appear to be almost additive, with no tendency toward synergism or antagonism. At the highest combination concentration for methylamine (20% ethylamine/80% methylamine), this point is above a straight line between the single chemical tests (100% ethylamine/0% methylamine and 0% ethylamine/100% methylamine), and is observationally suggestive of antagonism as defined by Roales and Perlmutter (1974a). This allows a qualitative evaluation of the combined effects. Statistical assurance can be derived from these data by determining

Table I Combined and single toxicity of ethylamine (EA) and methylamine (MA) using various solution compositions.

Solution *% EA*	*Composition* *% MA*	*Counts* ± *CI*
100	0	252439 31399
80	20	214375 39112
60	40	242388 52097
40	60	239051 35721
20	80	243686 31654
0	100	225878 77369

100% ethylamine and 100% methylamine corresponds to each chemical's EC_{50} value. Other chemical amounts are related to a fraction of the EC_{50} as indicated. The counts represented for each solution composition are the arithmetic average of 20 assays.

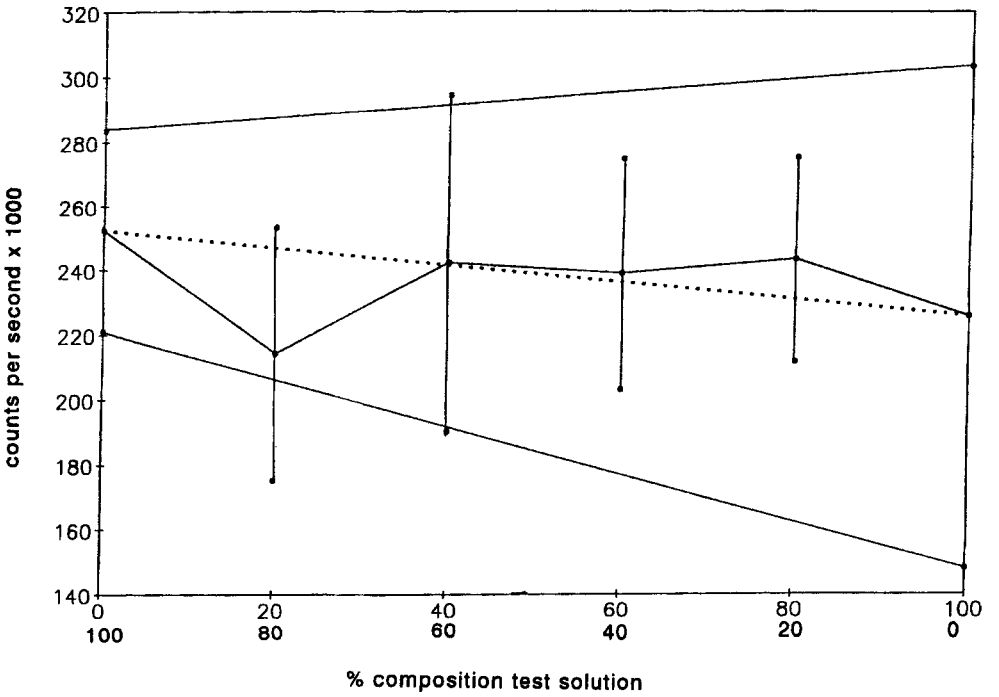

Figure 1 Isobole plot dosage-count curve for *Vibrio harveyi* bioluminescence after exposure to varying percentages of the EC_{50} concentration for ethylamine (EA) and **methylamine** (MA).

whether these mixture values on the plot are different from the straight line between single EC_{50} concentrations (100% and 0%) (Altenburger *et al.*, 1990; Thomulka and Lange, 1995). To evaluate these chemical combinations quantitatively in relationship to the straight line, a comparison of the CI for both the single experimental values and mixtures must be performed (Altenburger *et al.*, 1990). If an overlap exists between the CI values of the mixture and the individual components, no statistical difference is considered to exist. Using this criterion, if an overlap exists between the straight line and mixture, the mixture is considered not to deviate quantitatively from additivity. The CI around this straight line has been called the "confidence belt of the additivity line" and represents a method of determining statistical significance for mixture studies by Altenburger *et al.* (1990). Descriptively, the 20% EA + 80% MA mixture on the isobole plot is synergistic and the 80% EA + 20% MA is slightly antagonistic. The CI's for 100/0% EA and 100/0% MA overlap, suggesting that they do not vary from additivity based on a statistical difference. This illustrates the importance of including statistical techniques when evaluating mixture study data.

The isobologram, as described by Altenburger *et al.* (1990) for these data, are shown in Figure 2. Comparison of the CI for the individual chemicals, MA and EA, which are represented as their EC_{50}s and the CI for mixtures, suggest that all the combinations tested are qualitatively additive, with little or no synergistic or antagonistic tendency. Evaluation of CI overlap, as discussed for isobole plots, also suggest that these mixture combinations are quantitatively additive. These data support the previous isobole plot and Table I, further suggesting the additivity of these mixtures.

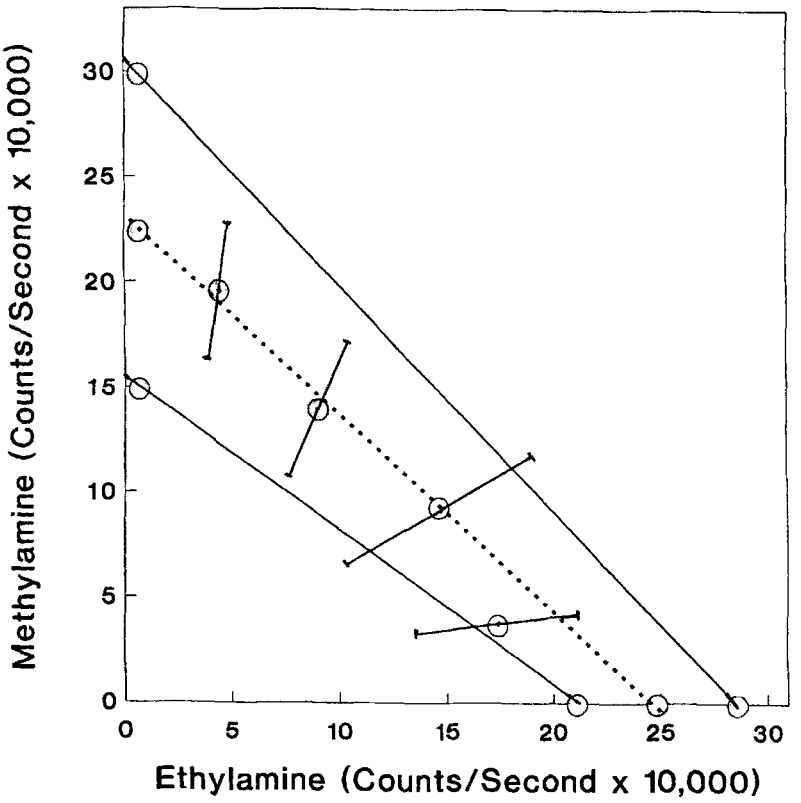

Figure 2 Isobologram plot of various combinations of ethylamine and methylamine EC_{50} concentrations for bioluminescence counts of *Vibrio harveyi.*

The additive index model, which uses the EC_{50} concentrations of each chemical together (EC_{50} concentration of EA and EC_{50} concentration of MA together in the same experimental reaction tube), suggests that these chemicals are synergistic using the *Vibrio* bioassay (Table II). The synergism for the combined EC_{50} concentrations may be a result of the high concentrations of these chemicals in the mixture. Concentrations of these chemicals at approximately equal mixtures but at individual concentrations of each chemical below its EC_{50} value, as seen for 40% ethylamine/60% methylamine and 60% ethylamine/40% methylamine, appear to have an additive effect using both the isobole plot and isobologram methods. Thus, the concentration of the chemical

Table II Evaluation of additive toxicity of ethylamine (EA) and methylamine (MA) and its range.

Mixture	*Additive Index*	*Range*
100% EA, 100% MA	1.65	1.63 – 1.67

Additive index is calculated using the additive index equation published by Marking and Mauck (1975). The index and range were determined from the arithmetic average of 34 assays. 100% corresponds to each chemical's EC_{50}.

mixture to which the test organism is exposed appears to be important for determining the outcome. Evaluation of these chemicals singly, at their EC_{50} concentration values, was found to have counts and a CI of 225878 ± 77369 for ethylamine and 252439 ± 31399 for methylamine (Table I). Inclusion of CI values for each chemical allows statistical comparison with other bioassay studies (Lange and Thomulka, 1993).

Since methylamine is descriptively more toxic, it is not surprising that at a higher mixture ratio (20% ethylamine/80% methylamine) synergism is observed qualitatively on the isobole plot. However, the qualitative occurrence of antagonism and a clear lack of synergism at the lower mixture ratio of methylamine with ethylamine is intriguing. All proportions evaluated using the isobologram method are quantitatively additive. No descriptive variation, including at the higher MA ratio, is observed in the isobologram. It is possible that ethyl and methyl groups have different sites of action on the bacterium or different kinetics of uptake at varying ratios as seen in many metabolic pathways (Moat and Foster, 1988). This, in part, could explain the different EC_{50} values, but appears to provide no clue as to the similar toxicity of the mixtures as indicated by the isobologram and the qualitative synergism seen on the isobole plot for 20% EA and 80% MA.

Statistical comparisons between the various interval groups are shown in Table III. Evaluation using the t-test suggest that the difference between the two extreme values, 80% ethylamine/20% methylamine and 20% ethylamine/80% methylamine are statistically significant. No other comparisons were statistically different at the 0.05 level for any of the other interval groups. The statistical datum (80% ethylamine/20% methylamine and 20% ethylamine/80% methylamine) supports the qualitative or descriptive interpretation of synergism and antagonism related to the isobole plots. However, caution must be applied since no statistical significance exists between the single component toxicity results (straight line between 100/0% ethylamine/methylamine and 0/100% ethylamine/methylamine) and either of these (80% ethylamine/20% methylamine or 20% ethylamine/80% methylamine) values. This lack of a statistical difference between the single chemical experiments, suggests that their EC_{50}s are statistically similar, although descriptively different. Comparison of the other interval groups, and

Table III Significance of difference between toxicants in the ethylamine (EA) and methylamine (MA) interaction comparison tests.

Combinations	*t value*	*Probability*
100% EA vs. 100% MA	1.215	n.s.
0% EA, 100% MA vs. 20% EA, 80% MA	0.754	n.s.
20% EA, 80% MA vs. 40% EA, 60% MA	0.371	n.s.
40% EA, 60% MA vs. 60% EA, 40% MA	0.204	n.s.
60% EA, 40% MA vs. 80% EA, 20% MA	0.226	n.s.
80% EA, 20% MA vs. 20% EA, 80% MA	2.229	$>0.02 < 0.05$
80% EA, 20% MA vs. 100% EA, 0% MA	0.470	n.s.

$N = 20$, $p = 0.05$ when $t = 1.729$
n.s. - not significant at 0.05

their lack of a statistical significance with the synergistic or antagonistic combinations, suggest that these terms of toxicity for this evaluation must be considered only descriptive. Thus, synergism and antagonism can be defined in either descriptive (qualitative) or statistical (quantitative) terms depending on the comparison of the combined mixture and each of the single components' (ethylamine and methylamine) toxicity. However, chance must be considered in any statistical or descriptive evaluation, with all conclusions, especially those having a descriptive characteristic, being tempered with caution.

When evaluating the procedure presented here to describe combined effects of chemicals, each method appears to exhibit some unique characteristics (Marking, 1985). This study, although limited in nature, suggests that these methods after quantification appear to be equally effective in predicting response. However, other techniques have also been proposed for evaluating chemical combinations or mixtures (Kraak *et al.*, 1993; Konemann, 1981) including evaluation of individual chemicals separately to determine combined interactions (Sokal and Rohlf, 1995).

When evaluating and conducting mixture toxicity studies various physical, chemical and biological characteristics of the assay must be considered (Ghosh and Doctor, 1992; Lange and Thomulka, 1993; Thomulka *et al.*, 1993b; Codina *et al.*, 1993). This is illustrated by comparing these data with a previous study (Thomulka *et al.*, 1992) that evaluated ethylamine and methylamine using this assay procedure. The previous investigation used frozen stocks of these chemicals and phosphate buffered saline, rather than stocks freshly prepared for each experiment, and using imidazole buffer, resulting in a lower EC_{50} than seen in this study. These slight differences in experimental protocol can have not only dramatic effects on comparative data for single chemicals, but can also greatly affect mixture studies due to the interaction between chemicals.

The unusual effect observed in this study of two chemicals at different concentrations having both synergistic and antagonistic effects on the same organism is interesting. Since the natural environment is often composed of numerous chemicals, additional studies on combination toxicity are warranted to understand the relationship between chemicals in the environment (Marking, 1985; Alabaster *et al.*, 1988; Thomulka and Lange, 1995). The use of a bacterial bioassay system, as described in this paper, will allow these more complex investigations to be made, without the difficulty associated with multi-cellular assay systems.

References

Alabaster, J. S., Calamari, D., Dethlefsen, V., Konemann, H., Lloyd, R. and Solbe, J. F. (1988) Water quality criteria for European freshwater fish. *Chemistry and Ecology*, **3**: 165–253.

Altenburger, R., Bodeker, W., Faust, M. and Grimme, L. H. (1990) Evaluation of the isobologram method for the assessment of mixtures of chemicals. *Ecotoxicology and Environmental Safety*, **20**: 98–114.

Codina, J. C., Perez-Garcia, A., Romero, P. and de Vicente, A. (1993) A comparison of microbial bioassays for the detection of metal toxicity. *Archives* of *Environmental Contamination and Toxicology*, **25**: 250–254.

Dutka, B. J. and Kwan, K. K. (1981) Comparison of three microbial toxicity screening tests with the Microtox test. *Bulletin of Environmental Contamination and Toxicology*, **27**: 753–759.

Daniel, W. W. (1991) *Biostatistics: a Foundation for Analysis in the Health Sciences*. John Wiley, New York, NY

Deneer, J. W., Seinen, W. and Hermens, J. L. M. (1988) Growth of *Daphnia magna* exposed to mixtures of chemicals with diverse modes of action. *Ecotoxicology and Environmental Safety*, **15**: 72–77.

Dirilgen, N. and Inel, Y. (1994) Effects of zinc and copper on growth and metal accumulation in duckweed, *Lemna minor. Bulletin of Environmental Contamination and Toxicology*, **53**: 442–449.

Frumin, G., Chuiko, G., Pavlov D. and Menzkova O. (1992) New rapid method to evaluate the median effect concentration of xenobiotics in hydrobionts. *Bulletin of Environmental Contamination and Toxicology*, **49**: 361–667.

Ghosh, S. K. and Doctor P. B. (1992) Toxicity screening of phenol using Microtox. *Environmental Toxicology and Water Quality*, **7**: 157–163.

Hathaway, G. J., Proctor, N. H., Hughes, J. P. and Fischman, M. L. (1991) *Proctor and Hughes' Chemical Hazards* of the *Workplace.* Van Nostrand Reinhold, New York pp. 278–279 and 381–382.

Konemann, H. (1981) Fish toxicity tests with mixtures of more than two chemicals: a proposal for a quantitative approach and experimental results. *Toxicology*, **19**: 229–238.

Kraak, M. H. S. Schoon, H., Peeters, H. M. and van Straalen, N. M. (1993) Chronic ecotoxicity of mixtures of Cu, Zn, and Cd to the Zebra mussel *Dreissena polymorpha. Ecotoxicity and Environmental Safety*, **25**: 315–327.

Kreitzer, J. F. and Spann, J. W. (1973) Tests of pesticidal synergism with young pheasants and Japanese quail. *Bulletin* of *Environmental Contamination* and *Toxicology*, **9**: 250–256.

Lange, J. H. and Thomulka, K. W. (1993) Evaluation of aquatic toxicity studies using three different statistical methods: reference to aquatic bacterial bioassays. *Fresenius' Environmental Bulletin*, **2**: 758–763.

Marking, L. L. (1985) Toxicity of chemical mixtures. In. *Fundamentals of Aquatic Toxicity*, (G.M. Rand and S.R. Petrocelli, eds.), Taylor and Francis, Bristol, PA.

Marking, L. L. and Mauck W. L. (1975) Toxicity of paired mixtures of candidate forest insecticides to rainbow trout. *Bulletin of Environmental Contamination and Toxicology*, **13**: 518–523.

Moat, A. G. and Foster, J. W. (1988) *Microbial Physiology,* John Wiley, New York, NY.

Ribo, M. J. and Kaiser, K. L. E. (1987) *Photobacterium phosphoreum* toxicity bioassay I: Test procedures and applications. *Toxicity Assessment: an International Journal.* **2**: 305–323.

Roales, R. R. and Perlmutter A. (1974a) Toxicity of methylmercury and copper, applied singly and jointly, to the Blue Gourami, *Trichogaster trichopterus. Bulletin of Environmental Contamination and Toxicology*, **12**: 633–639.

Roales, R. R. and Perlmutter A. (1974b) Toxicity of zinc and cygon, applied singly and jointly, to Zebra embryos. *Bulletin of Environmental Contamination and Toxicology*, **12**: 475–480.

Sokal, R. R. and Rohlf, F. J. (1995) *Biometry*, W.H. Freeman and Company, New York, NY.

Somasundaram, L., Coats, J. R., Rache, K. D. and Stahr, H. M. (1990) Application of the Microtox system to assess the toxicity of pesticides and their hydrolysis metabolites. *Bulletin of Environmental Contamination and Toxicology*, **44**: 254–259.

Spliid, H. and Torslov, J. (1994) Statistical analysis of joint toxicity in biological growth experiments. *Ecotoxicology and Environmental Safety*, **28**: 181–192.

Thomulka, K. W. and Lange J. H. (1994) Physical, chemical, and biological characteristics of *Vibrio harveyi*, a marine bacterium, in a bioluminescence-reduction bioassay. *Journal of Clean Technology and Environmental Science*, **4**: 57–77.

Thomulka, K. W. and Lange, J. H. (1995) Multiple toxicity of three metals, tributyltin chloride, dibutyltin dichloride and monobutyltin trichloride, using the marine bacterium *Vibrio harveyi* as the test organism. *Fresenius, Environmental Bulletin* (in press).

Thomulka, K. W., McGee, D. J. and Lange, J. H. (1992) Evaluation of organic compounds in water using *Photobacterium phosphoreum* and *Vibrio harveyi* bioassays. *Fresenius' Environmental Bulletin*, **1**: 815–820.

Thomulka, K. W., McGee, D. J. and Lange, J. H. (1993a) Use of the bioluminescent bacterium *Photobacterium phosphoreum* to detect potentially biohazardous materials in water. *Bulletin of Environmental Contamination and Toxicology*, **51**: 538–544.

Thomulka, K. M., McGee, D. J. and Lange J. H. (1993b) Detection of biohazardous materials in water by measuring bioluminescence reduction with the marine organism *Vibrio harveyi. Journal of Environmental Science and Health*, **28A**: 2153–2166.

Thomulka, K. W., Kriebel, J. R., Schroeder, J. and Lange, J. H. (1993c) Toxicity of various chemicals in a sand and water mixture using the marine bacterium *Vibrio harveyi* in a direct bioluminescence-reduction bioassay. *Journal of Clean Technology and Environmental Science*, **3**: 217–226.

Timko, J and Downie J. (1992) *Statistics on Software*, Software Labs, Culver City, CA.

Williams, P. L. and Burson, J. L. (1985) *Industrial Toxicology*. Van Nostrand Reinhold, New York 255 pp.

de Zwart, D and Slooff, W. (1987) Toxicity of mixtures of heavy metals and petrochemicals to *Xenopus laevis. Bulletin of Environmental Contamination and Toxicology*, **38**: 345–351.