ÉTUDE DE FAISABILITÉ POUR ÉVALUER L'APPLICABILITÉ DE TECHNIQUES BIOANALYTIQUES POUR LES REJETS D'EAU AQUEUX AFIN D'IDENTIFIER LA ZONE D'INFLUENCE

par

Robert Roy

mars 1985

SOMMAIRE

Ce rapport présente les résultats d'une étude d'applicabilité des biotests, pour l'évaluation des eaux de surface se trouvant dans la zone d'influence d'un rejet d'égout domestique. Pendant la période août-décembre 1984, des échantillons provenant du tronçon Montréal-Lanoraie du fleuve Saint-Laurent ont été évalués avec trois techniques bio-analytiques (i.e. bioaccumulation, résistance microbienne et mutagénicité) indicatives d'aggression insidueuses. Ces tests font partis d'une approche écotoxicologique intégrée, conçue et utilisée par les laboratoires du SPE à Longueuil.

La résistance microbienne aux antibiotiques a été détectée pour toutes les stations étudiées. Quatre stations, soit Rivière des Miles Iles, Rivière l'Assomption, L,émissaire et la première station en aval de l'émissaire ont démontré les valeurs les plus élevées. Le nombre d'analyses a été limité à cause de la lourdeur des techniques analytiques. Les résultats obtenus démontrent néanmoins que l'on peut recommander la poursuite de telles analyses. Un protocol simplifié ou automatisé devrait cependant être utilisé. Les quatre stations pré-citées, une station en amont ainsi qu'une autre plus éloignée en aval, devraient constituer les bases minimales d'un réseau d'étude.

L'activité mutagénique était très faible ou non détectable pour la majorité des échantillons, sauf l'émissaire. En deux occasions

sur cinq, l'eau de l'émissaire a révélé un potentiel de reversion mutagénique sur une des trois souches utilisées. Il est recommandé que ce test utilise un facteur d'activation mammalienne pour augmenter la sensibilité. Un réseau d'étude devrait être constitué d'au moins 3 stations, soit l'émissaire, une station en aval et une en amont.

L'étude sur la bioaccumulation a démontré un potentiel de biosorption d'effets toxiques pour trois stations: l'émissaire, un poste en aval, et un poste localisé dans le bras Nord du chenail. D'autres postes ont révélé un potentiel d'accumulation pres du seuil de détection. Pour les postes en rivière, l'action biodégradatrice a augmenté ce potentiel à des niveaux détectables. Nous recommendons qu'un échantillonnage futur de ces stations soit plus fréquent afin de mieux appréhender cette agression insidieuse potentielle.

Pour accroître la sensibilité du test de bioaccumulation, des raffinements protocolaires ont été intrepris. Il est recommandé que l'algue Chlamydomonas variabilis exposée à un volume d'échantillon d'un litre soit employée à cette fin. Ce protocole incorpore une étape oxydative afin de libérer les substances liées organiquement. On peut par la suite se servir du microtest algal et du test Microtox pour détecter les substances toxiques accumulées.

On recommande également en appendice comment ces bioessais peuvent être utilisés pour l'évaluation routinière d'eaux de surfaces et d'échantillons divers.

ABSTRACT

A study was undertaken to estimate the usefulness of specific biotests for the evaluation of surface waters. The aim of the project was to identify the area of impact, if any, of a municipal wastewater discharge on the Montreal - Lanoraie Sector of the St-Lawrence River, using bioanalytical parameters indicative of insiduous aggression.

Three bioanalytical techniques were employed: an algal bioaccumulation test, a measure of bacterial resistance to antibiotics, and a mutagenicity test derived from the Ames test. These tests from part of an integrated approach to ecotoxicological evaluation of wastewaters, which was conceived by EPS - Quebec Region.

Samples were collected during a 5 month period (August to December, inclusive). Analyses were performed on filtered samples both before and after a biodegradation treatment.

Bacterial resistance to antibiotics was detected for all stations. Four stations (i.e. Des Miles Iles river, L'Assomption river, the municipal discharge and a sampling station immediately downstream of the discharge) displayed the most resistance. Because of the laborious methodology, the number of samples was limited; yet the results indicate the need to continue similar analysis. It is recommended that the protocol of this test be automated or simplified to permit more extensive and frequent sampling of this region. A

minimal network should be composed of at least six sampling stations. This network should comprise the four stations previously stated an upstream stration and a station located further downstream.

The mutagenicity test did not detect effects for most samples, with the exception of the municipal wastewater. For this site one of the three bacterial strain used showed reversion on two out of five occasions. Further testing should incorporate the mammalian microsome activation factor to augment sensitivity. At the very least. additional sampling should include the municipal wastewater on upstream and a downstream station.

Bioaccumulation studies showed potential uptake of toxicity for three stations: the municipal wastewater, a downriver station and the North channel station. Other stations showed bioaccumulation potential near the threshold of detection. Biodegradation treatment increased this potential to detectable level for the river stations. It is recommended that future tested stations be sampled frequently to better assess this potential insiduous aggression.

To increase the sensitivity of the bioaccumulation test refinements in the protocol were attempted. The alga Chlamydomonas variabilis and 1 Liter sample volumes are suggested for this protocol. Options of an oxidation step to liberate organically-bound substances, and the use of the Microplate Algal Test, in addition to the Microtox test, to better detect and precise the nature of the accumulated toxic substances, is included.

General recommendations for routine use of the bioassays for surface waters and other samples are also presented in Appendices.

TABLE OF CONTENTS

LIST OF TABLES

TERMS OF REFERENCE AND AREA OF STUDY

This report deals with studies undertaken to determine the usefulness of specific biotests, commonly used by EPS Quebec region laboratories, for the evaluation of surface waters in view of identifying the zone of impact of wastewater discharges. This project was in the context of contract serial #OSD 84-00076: "Etude de faisabilité pour évaluer l'applicabilité de techniques bioanalytiques pour les rejets d'eaux aqueux afin d'identifier la zone d'influence".

The area of study was the Montreal-Lanoraie sector of the St. Lawrence River. Sampling sites were established by the Inland Waters Directorate; their location is listed in the NAQCENT station list (NAQUADAT, 1984) available from the Data Systems Section of the Water Quality Branch (Environment Canada).

ANTIMICROBIC SUSCEPTIBILITY TEST

Introduction:

The increasing occurrence of resistance to antibiotics by bacteria has become a potential risk to the control of infectious disease. One of the mechanisms of this resistance is the transfer of extrachromosonal resistance factors between bacteria, even between different strains. Bacterial resistance was monitored during the sampling programme to test the usefulness and sensitivity of this technique on surface waters.

The antimicrobic susceptibility test is one technique developed as a measurement of bacterial resistance. Pure bacterial cultures are isolated, inoculated on agar with antibiotic discs, and incubated overnight. Sensitive bacteria will exhibit a zone of inhibition (no colony growth) around the discs; a measurement of this zone (in mm) is a quantitative indication of bacterial resistance to antibiotics.

During this project, fecal coliforms were isolated from sampled stations; at least 20 colonies were then positively identified and recultured. These different cultures were then incubated with 12 antibiotic discs:

Triple sulfa	Tobramycin
Carbenicillin	Streptomycin
Kanamicin	Ampicillin
Colistin	Cephalotin
Tetracycline	Amikacin
Gertamycine	Chloramphenicol

After overnight incubation, the zone of resistance was measured and graded as sensitive, intermediate or resistant according to the type of antibiotic.

Results and discussion:

The results for each sampling station and sampling period are presented in Table I, expressed as a percentage of strains resistant to 3 or more antibiotics. In most cases, 20 strains were used; exceptions are noted. Values greater than background resistances (-15%) are considered significant.

Out of the 16 individual analyses, all stations revealed resistance to antibiotics. Two stations revealed marked antibiotic resistances (#9015 - 30% and #9070 - 37%). Two other samples had values approaching 30% - #9068 (25%) and the effluent (23%). As well, due to technical error, the first two sampling periods may have underestimated the resistance to the antibiotic triple sulfa and thus lowered the percentage of resistant strains.

Unfortunately the limited number of results precludes extensive discussion. Only 5 stations were sampled twice during this project; the difference between successive samples was sometimes a factor of 3, though still less than significant. Successive effluent samples differed by a factor of 2, perhaps due to real differences, perhaps due to normal sample variation. Nevertheless, the significant results obtained, do warrant continuation of this type of investigation on surface waters.

The technique used at present is laborious and may not permit extensive sampling. Two antibiotics (Gentamycin and Ampicillin) had sensitive responses 83% of the time; fecal coliforms were sensitive to colistin on 78% of the sample trials. These values may have elevated values due to the limited number of trials, yet the possibility exists that the bacterial response may also be constant. If such is the case, these antibiotics may be eliminated to simplify the protocol.

If the methodology remains unchanged, it would be more profitable to obtain more repeat samples of fewer stations to enable an estimation of the variability of samples. Meaningful comparisons between sampling periods and stations could then be made.

TABLE I

ANTIBIOTIC RESISTANCE OF FECAL COLIFORMS

(% OF 20 STRAINS RESISTANT TO 3 OR MORE ANTIBIOTICS)

SAMPLING STATION	DATE SAMPLED			
	JUNE 5/84	AUG 22/84	NOV 13/84	DEC 10/84
9013	-	15	-	-
9014	-	16 (a)	-	-
9015	-	30	-	-
9031	-	15	-	-
9039	6	20	-	-
9040	15	5	-	-
9041	0	15	-	-
9068	-	25	-	-
9069	10	10	-	-
9070	-	37 (b)	-	-
Effluent	-	-	23 (c)	12 (d)

(a) 16 strains
(b) 19 strains
(c) 38 strains
(d) 40 strains

MUTAGENICITY TEST

Introduction:

The Salmonella mutagenicity test, developed by Ames et al. (1975) and revised by Maron and Ames (1983), was designed to determine the mutagenic potential of samples. This plate test uses several mutant strains, which lack the capacity to manufacture the amino acid histidine and thus require it in the media. If incubated with mutagenic substances, these strains may undergo greater reversion to auxotrophy, compared to the spontaneous mutation rate of controls.

A microtitre fluctuation test was then introduced which allowed a statistical comparison between induced and spontaneous mutations, with greater sensitivity than the plate test. This variation has been adopted by this laboratory and uses these 3 Salmonella typhimurium strains:

TA 97 : detects frameshift mutations

TA 100: detects base pair substitution mutations

TA 102: detects oxidative mutants.

The protocol involves preparation of sample concentrations (based upon the results of toxicity tests); then incubation with the particular bacterial strain for a period of 3 days at 35°C. Revertants to auxotrophy were detected using a pH sensitive colour indicator; positive scores for control and sample plates were compared using the X2 test for significance. If mutagenic, the samples were retested within as short a delay as possible to verify the result.

Results and discussion:

Results are presented in Table II, expressed as Genotoxic Units (TUG), calculated as 100% divided by the minimum active dose (MAD). The MAD is defined as the concentration which results in a doubling of the spontaneous mutation rate; it is obtained from a linear regression of concentration and induced mutation rate.

The first set of samples were tested with 3 strains (TA 97, TA 100 and TA 102); the second with 2 (TA 97 and TA 100). The second set of samples were retested due to weak bacterial growth; this revealed abnormally high reversion rates for both TA 97 and TA 100 and indicated unstability. All three strains were therefore checked for maintenance of genetic markers: TA 100 and TA 102 were found to have lost plasmid coded resistance to antibiotics. These strains were then re-isolated and new master cultures prepared; later tests were all done with these 2 strains.

During the first 2 sample series, toxicity tests were performed prior to mutagenicity tests, according to the standard protocol. These mutagenicity tests used concentrations ranging up to 100%v/v, few samples gave positive responses at these concentrations due to toxicity. After this period, only mutagenicity tests using concentrations $< 25\%$ were done, immediately upon reception of the

sample. While most of the data indicate non detectable at these lower concentrations, previous results demonstrate no mutagenic activity above 25%v/v.

These results in Table II are those for strain TA 102, with one exception (August 6th samples - TA 100). TA 102 was the only strain to elicit any positive responses during this project, with the exception of one sample.

Verification of a mutagenic response was done within the shortest possible time, usually the same day as results were scored. Certain samples had variable results, including those of other projects (data not shown). If duplicate tests were run at the same time, this problem did not arise. All testing after the September 24 series was done in duplicate for greater reproducibility.

Mutagenic activity was detected on only 7 occasions, two of these being effluent samples. On one occasion the biodegraded sample was non-mutagenic (Sept 4 series); while the Sept 24th sample became mutagenic after this treatment. Thus biodegradation treatment may significantly alter the genotoxic properties of the sample.

The interpretation of the biodegradation results is somewhat difficult; this treatment is itself of variable mutagenicity, as can be seen in Table III. The control for biodegradation should be investigated more thoroughly to determine the cause of this effect (ie: nutrient supplement causing increased numbers of bacteria or a real mutagenic effect).

The main conclusion of this study is that none of the strains used were sensitive enough to detect potential mutagens in surface waters. Even TA 102, the most sensitive of the 3 strains used, gave positive results on 5 of 48 samples. Therefore, any future mutagen testing of surface waters should incorporate S-9 or a similar mammalian microsome activation system into the test. This would enhance the sensitivity of the test, as well as facilitating the appraisal of risks to human health.

Any future project involving this region should definitely include the effluent among the samples tested, due to the mutagenicity (24 to $>$ 62 TU_G) found in two samples. As may as possible of the other sampling sites should also be re-rested as frequently as is judged necessary if the effluent mutagenicity is recurrent.

TABLE II

MUTAGENICITY (TUg)

# OF STATION	MAY 17/84		AUG 6/84	BIOD	SEPT 4/84	BIOD	SEPT 24/84	BIOD	OCT 22/84	BIOD	NOV 14/84	BIOD
9013	-		-	-	-	-	ND	-	-	-	-	-
9014	-		-	-	ND	-	ND	ND	-	-	-	-
9015	-		-	-	ND	ND	ND	ND	-	-	-	-
9016	-		-	-	-	-	ND	-	-	-	-	-
9017	-		-	-	-	-	ND	-	-	-	-	-
9027	-		-	-	-	-	ND	-	-	-	-	-
9031	-		ND,	-	4.0, ND	ND	ND	ND	-	-	-	-
9039	4.0		ND,	-	ND	ND	ND	ND	-	-	-	-
9040	ND		ND,	-	ND	-	ND	ND	-	-	-	-
9041	10.5		2.0	-	ND	-	ND	ND	-	-	-	-
9068	-		-	-	-	-	ND	-	-	-	-	-
9069	11.0		ND,	-	ND	-	ND	ND	-	-	-	-
9070	-		-	-	-	-	ND	-	-	-	-	-
EFFLUENT:	-		ND,	-	30, 62	ND	ND	50, 24	ND	ND	ND	ND

ND = not determined at concentrations tested
BIOD = sample after biodegradation step
All results are for strain TA 102, except for AUG 6 series (TA 100)

TABLE III

MUTAGENICITY OF BIODEGRADATION CONTROLS

(TU_g)

DATE	STRAINS		
	TA 97	TA 100	TA 102
July 7/84	N.S.	N.S.	N.S.
July 23/84	N.S.	N.S.	N.S.
Nov 9/84	N.S.	N.S.	1-2
Nov 19/84			
3 days incubation:	-	-	4-8
4 days incubation:	-	-	8

BIOACCUMULATION TEST

Introduction

The bioaccumulation test was designed to estimate the potential for increased toxicity due to the accumulation of substances from a sample. It involved two organisms - one as the bio-concentrator (Selenastrum capricornutum, a fresh water alga routinely used in enrichment and inhibition tests), and another as the detector of toxicity (The Microtox" bacterial system). The rationale was that an alga, incubated with a sample, would possibly accumulate toxic substances which could then be detected by the "Microtox".

The methodology used in following tests involved the dilution of a 4 day old algal culture (in the log phase of growth), with an equal volume of sample to arrive at an innoculum of 2 X 10^6 algal/cells/mL. After 24 hours of contact, a cell count was made, the test volume was centrifuged and the agal pellet recouperated. The cells were lysed by ultra-sound and the lysate volume brought to 3 mls with sterile $NaHCO_3$. The lysate was subsequently filtered and the supernatent collected for toxicity testing.

Certain details of the above protocol were modified according to laboratory conditions at the time of testing. In some cases, the algal cultures were centrifuged to arrive at an innoculum of 2 X 10^6

cells/mL. To increase the sensitivity of the test, the test volumes were increased to 200 mls, then to 500 mls, while maintaining the dilution of 50%v/v between sample and algal culture. The final sample tested involved the addition of a concentrated algal innoculum to 1L of sample. Tests involving charges in test volumes are indicated in Table V.

For the first series of bioaccumulation tests, (July 4, 84), the lysate was filtered by a 0.45u membrane filter. The inference that lysate colour and/or chlorophyll content were unduly interfering with supernatant toxicity led to the decision to filter the lysate through a 0.2u filter.

Results and discussion:

Results for 100 and 200mL test volumes are presented in Table IV and include results for biodegraded samples. The calculation of sub-lethal Toxic Units/g of algae (TUsl/g) is based upon a 100mL sample (200mL test volume) and was determined from the following formula:

$$\left(\frac{TU_S - TU_C}{mL}\right) \frac{x\ 3mL\ x\ 1000mg/g}{mg\ algae}$$

where TU_S = toxic units of 1mL sample lysate (Microtox volume tested)

TU_C = toxic units, average control lysate, per mL

mg algae = mg. of algal concentrate in 3mls; determined from cell count at end of test

The cell count to biomass conversion is based on experimental determination of the weight of Selenastrum capricornutum: 100×10^6 = 1.0mg.

For the first series of tests (Aug. 4, 1984), no cell counts were done. The biomass was thus estimated from the initial innoculum (2mg/100mL test volume; 4mg/200mL test volumes). The TUsl/g of 100mL test volumes were subsequently doubled to arrive at TUsl/g for 100mls of sample (=200mL. test volume). One test (Oct 22 effluent duplicates)

involved volumes of 200 and 250mls; both results were non-determinable (ND) after a colour correction test.

Average control values were obtained from 30 similar trials; 100 and 200mL test controls overlapped considerably and were grouped together. The non-determined values were considered as equal to 1.0 T.U. which may lead to a slight overestimate of the average control. The calculated average was 1.8 T.U. Statistical testing of these controls revealed deviations from a normal distribution, which may indicate the measurement of several effects at once.

Sample TUs1/g were for these tests were considered significant if they were greater than the highest control value (1200 TUs1/g); this was obtained using the control biomass, control lysate T.U. and the average control T.U. (1.8).

For 100mL and 200mL test volumes, 18 out of 49 samples tested were significantly different than controls, according to the above criteria. Biodegration of the sample increased the toxicity significantly on 5 occasions; twice for stations #9014 and #9030 and once for station #9040. This may indicate increased bioavailability of toxic substances after biodegradation. If averages of duplicate tests are considered, the effluent remained relatively constant, with one exception. The Nov 13th sample value is significantly reduced after biodegradation; bioaccumulable substances were possibly degraded to non-toxic forms.

Results for test volumes of 500 to 1000mls are presented in a separate table (Table V), as the algal recooperation step was somewhat different. These cultures were pressure filtered, using a larger 0.2u filter; the filter was subsequently scraped to collect the algae. Colour correction tests were also performed to eliminate some of the interference with toxicity.

Statistical analysis was done to correlate control biomasses with their toxicity and to estimate control values for these greater biomasses. Unfortunately, no correlation was found between biomass and toxic units.

However, if controls for each test are used, the Dec 10th effluent sample showed potential for bioaccumulable toxicity. This toxicity is not present in the biodegraded sample; a similar occurrance to the Nov 13th effluent samples. The bioaccumulable substances are transformed during biodegradation of the effluent and become less toxic. It is possible that the effluent showed increased potential for bioaccumulation over time.

Overall, the results indicate significant bioaccumulation for stations #9014 (downriver), #9030 (upriver channel) and the effluent. The latter had significant bioaccumulation valves for 5 of 6 non-biodegraded samples, if the limit is considered as 900 TUsl/g.

Though this is somewhat less than the greatest control, it is a value higher than the "average" control. Generally, many samples (23/49) had values at or near this value and were near the limit of detection. A more sensitive test may have revealed this potential and the programme to develop such a test is discussed in the Appendix.

TABLE IV

BIOACCUMULATION TUs1/g ALGAE FOR 100mls OF SAMPLE

# OF STATION	AUG 6/84	BIOD	SEPT 4/84	BIOD	SEPT 24/84	BIOD	OCT 22/84	BIOD	NOV 13/84	BIOD
9013	75	-	-	-	-	-	-	-	-	-
9014	1050	-	882	1714 *	1500 *	5226 *	-	-	-	-
9015	3225 *	-	488	656	(294)	750	-	-	-	-
9016	3000 *	-	-	-	-	-	-	-	-	-
9017	3300 *	-	-	-	-	-	-	-	-	-
9027	900	-	-	-	-	-	-	-	-	-
9031	19500 *	-	ND	282	(276)	316	-	-	-	-
9039	34200 *	-	ND	1452 *	(702)	2057 *	-	-	-	-
9040	28200 *	-	ND	2444 *	136	871	-	-	-	-
9041	2775 *	-	ND	600	ND	818	-	-	-	-
9068	ND	-	-	-	-	-	-	-	-	-
9069	ND	-	-	-	-	-	-	-	-	-
EFFLUENT	2400 *	-	1059	(1488)	(1262)*	1318 *	(ND)**	-	3133 *	250

* significant value
**colour effect eliminated

Averages of duplicate tests are shown in brackets.

TABLE V

BIOACCUMULATION RESULTS FOR TEST VOLUMES OF 500 AND 1000 MLS

DATE OF SAMPLE	SAMPLE	MICROTOX IC50 (TU) colour correction	
		Non-Adjusted	Adjusted
Oct 15, 1984 (500mL test vol.)	Effluent after biodegradation	12.3	4.5
	Control	7.1	4.7
Dec 10, 1984 (1000mL test vol.)	Effluent**	10.4	8.5
	Control	6.8	6.0*
Dec 10, 1984 (1000mL test vol.)	Effluent** after biodegradation	2.0	-
	Control	2.7	

* colour correction done by eliminating highest concentration (50%v/v) in calculation of IC50

**little or no growth during test, as determined from innoculum and final cell count.

ANTIBIOTIC SUSCEPTIBILITY TEST

Conclusion and Recommendations

The variation in the results of this project make definitive interpretation difficult, yet the appearance of certain high values may indicate environmental problems. Therefore, it is recommended that antibiotic resistance testing be continued in future sampling programmes if resources permit.

It would also be preferable to obtain much more data for each station for reasons discussed earlier. Unfortunately, given the time consuming protocol of this assay, it may be preferable to limit the number of stations sampled yet increase the frequency if the protocol is unchanged. This would in turn limit the area of investigation or amount of information. Thus, it is recommended that the protocol be simplified or automated to increase the area of investigation and the number of samples per station.

MUTAGENICITY TEST

Introduction:

The Salmonella mutagenicity test, developed by Ames et al. (1975) and revised by Maron and Ames (1983), was designed to determine the mutagenic potential of samples. This plate test uses several mutant strains, which lack the capacity to manufacture the amino acid histidine and thus require it in the media. If incubated with mutagenic substances, these strains may undergo greater reversion to auxotrophy, compared to the spontaneous mutation rate of controls.

A microtitre fluctuation test was then introduced which allowed a statistical comparison between induced and spontaneous mutations, with greater sensitivity than the plate test. This variation has been adopted by this laboratory and uses these 3 Salmonella typhimurium strains:

TA 97 : detects frameshift mutations

TA 100: detects base pair substitution mutations

TA 102: detects oxidative mutants.

Future sampling of this sector would include as many stations as possible in this area, especially if a more responsive test is used. The effluent is the most important of these stations, due to its mutagenic activity on two occasions. The stations above Lanoraie (#9027, 9016 and 9017) may be excluded; ideally all other stations should be included, at the very least additional sampling should include the effluent an upstream and a downstream station. This programme should also include several samples over time, to follow the variation of responses due to seasonal or other effects.

BIOACCUMULATION

Conclusions and Recommendations

The results of this sampling programme indicated the need for further refinements in the protocol to increase the sensitivity and reproducibility of this test, especially with regard to surface waters. Some of the stations sampled (#9014, #9015, the effluent), exhibited a tendency to accumulate substances, even if the test used had some weaknesses.

Any future project involving this sector should include 10 sampling stations (omitting those above Lanoraie) plus the municipal effluent. This would ensure sufficient data for the environmental assessment of this region. These should be tested as often as practical to obtain estimates of variation for each sampling station. It is also recommended that tests on biodegraded samples be included, as there is a greater potential for increased expression of accumulated substances (toxicity) after this step for river samples.

Individual tests should use 1L. of sample to maximize this potential, and they should be performed in duplicate. The bioaccumulation test used had important differences between duplicate

sample values expressed in TUsl/g; this may indicate the possibility of many false negative or false positives. Duplicate tests would therefore quantify the variation in tests, and may increase confidence levels for the results obtained.

The bioaccumulation test to be used in future tests is a revised one; it utilizes the algae Chlarydomonas variabilis and has the option of an oxidation step (to remove chlorophyll and colour, yet liberate organically bound substances). As well, it is possible to use the Microplate Algal Test, as well as the Microtox, to determine the toxicity of bioaccumulated substances. This test is more fully described in the Appendix.

APPENDIX

APPENDICE I

PROTOCOLE DU TEST ANTIBIOGRAMME

Principales étapes:

1. Dénombrement des E. coli sur géloses mFC Difco préalablement incubées 18 à 24 heures à 44.5°C.

2. Prélèvement des colonies E. coli bleues et bleues-grises sur géloses mFC pour purification et confirmation sur géloses EMB Oxoid, incubation à 37.5°C pour 18 à 24 heures.

3. Prélèvement des colonies typiques ayant un reflet vert métallique et/ou un centre noir des géloses EMB pour transfert sur géloses nutritives inclinées en éprouvette, incubation de 18 à 24 heures à 37.5°C.

4. Préservation des souches typiques à 4°C si nécessaire.

5. Réanimation et/ou fortification des souches isolées par inoculation de bouillons nutritifs Difco, l'inoculum doit être très léger, incubation de 18 à 24 heures à 37.5°C.

6. Dilution des bouillons nutritifs inoculés ou ensemencés à raison de 1mL dans 9mL d'eau stérile tamponnée.

7. Ensemencement des géloses DST d'Oxoid dans des assiettes de Pétri de 150 x 15mm par inondation avec les dilutions effectuées en 6.

8. Les géloses DST sont décantées, inversées, et laissées à sécher pour 2 heures à la température de la pièce.

9. Application des disques d'antibiotiques à l'aide d'un distributeur automatique Difco. Appuyer sur les disques à l'aide d'une pincette stérile pour bien faire adhérer ceux-ci. Laisser reposer les géloses 5 minutes à l'endroit avant de les inverser pour l'incubation à 37.5°C pour 18 à 24 heures.

10. Mesure en millimètres du diamètre des zones de réaction aux antibiotiques des souches E. coli testées. Les réponses sont classées en trois groupes: sensible, intermédiaire et résistante, selon la mesure obtenue. Le nombre de souches typiques ayant résisté à trois antibiotiques ou plus est rapporté. Ces souches de E. coli sont caractérisées comme offrant une résistance de nature plasmidique.

APPENDICE II

MUTAGENICITY PROTOCOL

MICROTITRE FLUCTUATION TEST

A fluctuation test measures the reversion to amino acid independance by a bacterial strain, thereby also quantifying the mutagenic effect of chemicals or effluents. A strain is selected which requires an amino acid in the growth medium. It is then incubated with concentrations of the sample and a small quantity of the required amino acid. This allows some growth of the cells until a mutation can be expressed. The appearance of a mutant colny can be indicated by a colour change in the media, the result of a change in pH.

Bacterial strains also undergo spontaneous mutation. Using the "microtitre" method, the fluctuation test allows for a statistical comparison between the induced and spontaneous mutations, with greater sensitivity than previous tests.

One of these tests was developed by Ames et al (1975), and involves various mutant strains of the bacteria Salmonella typhimurium.

These mutant strains all require the amino acid histidine, in contrast to the wild type:

TA 97: detects frameshift mutations

TA 98: " " "

TA100: detects base pair substitution mutations

TA102: detects oxidative mutants.

If incubated with small amounts of histidine in the media, some revertants back to the wild type will be observed (=spontaneous mutants). If a mutagen is also present, it should produce more mutations than the control series. The revertant colonies in this test are easily identified with the use of the indicator dye bromthymol blue.

The stock cultures are kept in an ultra-low freezer (-80°C) to minimize reversion. A test culture is prepared by taking a loopful of stock culture and placing it in 5mls of nutrient broth; this is incubated overnight in a water bath. The next morning a toxicity test is performed to determine sample concentrations which allow bacterial growth, and therefore the occurrence of mutations. Then, again using an overnight culture, a fluctuation test is done to determine the mutagenicity of the sample.

PREPAPATION OF SAMPLE

The sample pH should be adjusted to pH 7-8, then the sample should be filter sterilized through a 0.2u membrane and kept at 4°C until tested.

PREPARATION OF MATERIALS

Sterilize sufficient quantities of pipets, graduated cylinders, plastic pipet tips, test tubes, etc., for all tests.

PREPARATION OF OVERNIGHT CULTURE

In the late afternoon, take a loopful of stock culture and innoculate in 5mls of nutrient broth; this will prepare a culture for testing the next day. Place in shaking water bath at 37°C overnight.

PRELIMINARY TOXICITY TEST

The strain is incubated with concentrations of the sample (100%, 50%, 25%...0.1%) in a microtitre plate. Each plate covers a range of 11 concentrations plus a control, with 8 replicates each.

METHOD

Prepare fluctuation media: concentrated media for effluent samples, dilute media for purified or concentrated chemicals. If using dilute media, the overnight Salmonella culture must also be diluted the morning of testing (1mL culture into 9mls nutrient broth).

At least 3mls of media are required for each plate (96 wells/plate x 0.03mL/well = 3mls), but usually 5mls are prepared. Add sufficient bacteria from the overnight culture to the media for a final density of 2 x 10^5 cells/mL media. Mix thoroughly. Test strains with different innocula to determine reasonable revertant growth in the controls. Prepare the sample dilutions. Approximately 2mls are required per dilution (0.2mL/well x 8 wells - 1.6mls/dilution)

but usually 5mls/dilution are prepared. Mix thoroughly also.

Add 0.2mL of each concentration to the 8 wells in the plate, starting from the control. (Use a repeating pipet). Then add 30uL of the innoculated media to each well of the plate.

Seal the plates in plastic bags and incubate at 35°C for 3 days.

RESULTS

(-) clear blue wells: toxic (no growth)

(-) clear blue-green wells: slight growth

(-) clear green wells: normal growth

(+) turbid green wells: late mutagenic response

(+) turbid yellow wells: mutagenic response.

FLUCTUATION TEST

Determine the concentrations for the fluctuation test from the toxicity results. Choose 5-6 concentrations covering the range from slightly toxic to no response. Each plate will receive one concentration of the sample only; thus 5 concentrations will require 5 plates + 1 control plate. The strain chosen will be incubated for 3 days; control and sample plate positive responses will then be compared using the chi-squared test for significance.

METHOD:

Prepare the fluctuation media: (20mls per strain) (30uL/well x 96 wells/plate x 6 plates = 20mls). If using more than one strain, divide the media accordingly, after preparing it. Mix thoroughly, then add sufficient bacteria from overnight culture to the media.

Prepare the dilutions using sterile 50mL tubes. Twenty (20) mls per concentration are required for each strain (ie: 0.2mL/well x 96 wells/plate - 20mls). Mix thoroughly, then add 3mls of innoculated media to each 30mL sample dilution.

Starting with the control, pour a dilution tube into a sterile dilution tray. Using a multipoint pipet, (200uL per well) fill all the wells in one plate. Repeat for each dilution, going from lowest to highest.

Seal the plates in a plastic bag and incubate for three days.

RESULTS:

Count positive responses (see toxicity results) and use X^2 test for significance.

$$\frac{X2 = Zn\ (t - c)\ 2}{(t + c)\ (Zn - t - c)}$$ with 1 degree of freedom

n = total # of wells in plate

t = total # of positive wells in treated plate

c = total # of positive wells in control plate.

X2 3.84, significance level = 5%

X2 6.64, " " = 1%

X2 10.83, " " = 0.1%

STOCK CULTURES: PREPARATION

Grow up an overnight culture in nutrient broth. Add DMSO to a final concentration of 10%. Put 1.8mls into stock culture vials, freeze at -80°C.

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Gatehouse, D. (1978). Detection of mutagenic derivations of cyclophosphamide and a variety of other mutagens in a "microtitre" Fluctuation test, without microsomal activation. Mut. Res. 53, 289-296.

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MEDIA

NUTRIENT BROTH

Oxoid Broth # 2	25g/L
d H_2O	1000mL
Agar (if required)	15.0g

Mix and autoclave.

CONCENTRATED FLUCTUATION MEDIA

5.5A Davis Mingoli Salts	72.8mL
40% Dextrose	16.0mL
Bromthymol blue (6mg/mL)	8.0mL
Biotin (0.1mg/mL)	
Histidine (0.05mg/mL)	4.0mL
	(100mls)

DILUTE FLUCTUATION MEDIA

4 X A Davis Mingoli Salts	12.5mL
40% Dextrose	2.0mL
Bromthymol blue (6mg/mL)	1.0mL
Histidine (0.05mg/mL)	
Biotin (0.1mg/mL)	0.5mL

Make up to 100mL with sterile dH_2O

May be stored at room temperature.

HISTIDINE - BIOTIN

Histidine:	5mg
Biotin:	10mg
Sterile d H_2O	100mL

Filter sterilize and store at 4°C.

DAVIS - MINGOLI SALTS

	4A	5.5A
K_2HPO_4	28.0g	38.5g
KH_2PO_4	8.0g	11.0g
$(NH_4)_2SO_4$	4.0g	5.5g
Tri-sodium citrate	1.0g	1.4g
$MgSO_4$ $7H_2O$	0.4g	0.55g
d H_2O make up to:	1000mL	1000mL

Mix and autoclave.

GLUCOSE MINIMAL AGAR (2.0%)

For 150 plates at 20mL/plate (1.5L of solution)

d H_2O	750mL
Difco Agar	22.5g

Heat with stirring, in 2L flask, until suspension is made. Autoclave.

d H_2O	650mL
Vogel-Bonner Salts 50XE	30mL

Mix in a 2L flask and autoclave.

d H_2O	100mL
Dextrose	30g

Mix and autoclave.

1. Cool all solutions to 50°C. Add glucose to salts, then to agar.
2. Add amino acids (Histidine and Biotin) for a final concentration of 10ug/mL ie: for 1.5L, add 15mg each of Histidine and Biotin to warm solution.

VOGEL - BONNER SALTS 50XE

d H_2O	670mL
$MgSO_4 . 7H_2O$	10.0g
Citric Acid	100g
K_2HPO_4	500g
$Na (NH_4) HPO_4 . 4H_2O$	175g

Add salts slowly to H_2O in order.

Add 1.0mL chloroform and shake.

No need to autoclave. Store at room temperature.

SODIUM AZIDE STANDARD (TA 100)

$Na N_3$	100mg
d H_2O	100mls

MUTAGENESIS TESTING

Additions to General Protocol

Upon reception, the sample pH should be adjusted to between 7.0 - 8.0. The sample should then be fitter sterilized through a 0.2u membrane and kept at 4°C in darkness. Testing should be done as soon as possible.

Overnight cultures should be prepared by taking a loopful of frozen stock culture and innoculating 5mls of nutrient broth. Sterile technique is imperative to avoid contamination of the stock culture; return the frozen master to -80°C as soon as possible. The overnight culture will be used for testing the next day. This culture should be placed in a 37°C shaking water both connected to a timer which is set for 12 hours operation. (ie: 20:00h - 08:00h). This should ensure a healthy culture with more constant cell numbers.

The next day, place the culture at 4°C until ready to be used (no more than 2-4 hours). Prepare the media as per the detailed protocol instructions.

If a toxicity test is to be performed (unknown effluents, pure chemicals), it is recommended that only 3-4 log concentrations

(plus a control) be tested per strain. (ie: 100%, 10%, 1%, 0.1%; using 2-3 columns per concentration on the same plate.

Alternatively, especially for surface water samples, 7 concentrations may be chosen: 100%, 50%, 25%, 12.5%, 6.25%, 3.1% , 1.6%. These concentrations should be sufficient to obtain calculable results. The higher concentrations may be omitted if necessary when similar samples are tested later.

For mutagenesis tests, dilutions should be prepared (in duplicate) and the bacteria added to the media (2-3ul of bacteria per mL media). After mixing well, add the bacterial suspension to each dilution tube. Mix the dilutions and plate them using the multipoint pipet (200uL/well).

The plates should be sealed in plastic bags, incubated for 3 days at 35°C, then scored. If results are in conclusive, reincubate the plates another day. Each duplicate should be done, and scored, as if it were a spearate test entirely. This will ensure verification of positive results.

Calculation of the MAD on the computer uses a linear regression programme. Eliminate toxic or non-significant results from those printed out before calculation of the MAD to ensure a high statistical correlation between induced mutation rate and concentration.

MAINTENANCE OF STOCK CULTURES/POSITIVE CONTROLS

For preparation of stock cultures, refer to the detailed protocol. Maintenance of genetic markers should be checked regularly, especially with TA 102, as this strain is unstable and contains two plasmids. The detailed protocol can be found in Maron and Ames (1983). If control spontaneous mutation rates are regularly unusually high (40-50) with the same quantity of innoculum, or if a period of months has elapsed between tests, it is suggested that the strains be checked and re-isolated if necessary.

Positive controls are suggested as standard protocol. Unfortunately, neither H_2O_2 not formaldehyde induce full reversion of TA 102 at non toxic doses in the fluctuation test. Other chemicals are extremely dangerous and require extensive laboratory equipment and care for safe use and disposal. In view of the limited screening role for this test, it is recommended that positive controls be dropped for TA 102 routine use. If TA 100 is used, lug/mL of sodium azide is a sure inducer of a 100% positive response.

APPENDICE III

BIOACCUMULATION ROUGH DATA & PROTOCOL I

PROTOCOL I

Four to five days before a bioaccumulation test, a culture of Selenastrum capricornutum was started with the following conditions:

Innoculum:	20,000 cells/mL
T°:	22-24°C
Volume of culture:	200mls; in a 1L flask
Media:	2 X AAP
Agitation:	manually, twice a day

Before a test, a cell count was performed on the culture to ensure that there were 4 X 10^6 cells/mL. If not, the culture was either diluted or concentrated by centrifugation (20 mins at 1500 g) to obtain this figure. A test was then started by combining 100mls of algal culture with 100mls of filtered (on 0.2u) sample. A control culture was similarly prepared by dilution in 100mls of buffered dH_2O. Following an overnight incubation period (>16 hours), the cultures were counted and then centrifuged for 20 minutes at 1500g s. The algal pellets were recouperated by decanting the supernatant, the pellets were then resuspended in $NAHCO_3$ buffered dH_2 (b H_2O), combined, and recentrifuged. The concentrated cell volume was brought up to 2mls with bH_2O to prepare for cell lysis on a Bronson Model IIc sonifier. Before addition of cells, the chamber was first rinsed with acid (10% HCl), then rinsed several times with sterile dH_2O. Sonification was carried out in the water-cooled chamber for twenty minutes at the following settings:

pulse sonification

50% duty cycle

output control: 4

Efficiency of cell lysis was regularly cheeked by microscopic examination and was 80-90%.

The lysate volume was broughjt up to 3mls with bH_2O, then filtered on a prewashed 0.45u filter* (washed with sterile dH_2O). The filtrate was collected, the salt concentration was adjusted to2% with NaCl; and a "Microtox" assay was performed.

The Microtox control and sample lysate toxicity results were then compared for significance.

---

*this procedure was modified, as of Sept 8/84, to filtration on a pre-washed 0.2u filter.

BIOACCUMULATION PROTOCOL I (Selenastrum capricornutum

200mls of Sel. cap. culture in 2 X APP, containing 4 x 10^6 cells/mL

↓

Separation into 2 x 100mL cultures

↓

Dilution with 100mL filtered sample or buffered dH_2O (control)

↓

Incubation for 16 hours (overnight)

↓

Recuperate algae by centrifugation (20 mins at 3000rpm)

↓

(Repeat centrifugation of pellets)

↓

Bring volume of pellets to 2mls with buffered dH_2

↓

Lysis by ultrasonifier

↓

Bring volume of lysed cells to 3mls buffered dH_2O

↓

Filter lysate on 0.45u; collect filtrate

↓

Test filtrate on Microtox

BIOACCUMULATION TEST RESULTS

- all test volumes = 200mls except (*) = 100mls
- TU_{s1}/g for 100ml of sample (200mL test volumes)
  values of TU_{s1}/g for 100mL tests were doubled for purposes of comparison
- biodegraded samples = B

DATE OF SAMPLE: AUG. 6, 1984

Station #	Total cell count (X10^6)	Biomass (mg)	Microtox IC50 (TU)	TU_{s1}/g
9013	-	4.0	1.9	75
9014	-	4.0	3.2	1050
9015	-	4.0	6.1	3225
9016	-	4.0	5.8	3000
9017	-	4.0	6.2	3300
9027	-	4.0	3.0	900
9031	-	2.0*	8.3	19500
9039	-	2.0*	13.2	34200
9040	-	2.0*	11.2	28200
9041	-	4.0	5.5	2775
9068	-	4.0	ND	-
9069	-	4.0	1.6	-
9070	-	4.0	ND	-
Effluent	-	2.0*	2.6	2400

DATE OF SAMPLE: SEPT. 4, 1984

TU_{s1}/g for 200mL TEST VOLUME

Station #	Total cell count (X106	Biomass (mg)	Microtox IC50 (TU)	TU_{s1}/g
9014	340	3.4	2,8	882
9015	432	4.3	2.5	488
9031	340	3.4	1.7	ND
9039	328	3.3	1.8	ND
9040	330	3.3	ND	ND
9041	380	3.8	ND	ND
9069	333	3.3	1.5	ND
Effluent	257	2.6	3.0	1059
9014B	280	2.8	3.4	1714
9015B	320	3.2	2.5	656
9031B	288	2.9	2.3	183
9030B	312	3.1	3.3	1452
9040B	273	2.7	4.0	2444
9041B	352	3.5	2.5	600
9069B	288	2.9	1.8	ND
Effluent B	296	3.0	1.4	176
duplicate tests	376	3.4	2.6	

DATE OF SAMPLE: SEPT. 24, 1984

Station #	Total cell count (X10^6)	Biomass (mg)	Microtox IC50 (TU)	TUs1/g
9014	344	3.4	3.5	1500
9015 duplicate	456	4.6	2.7	587
tests	232	2.3	1.4	ND
9031	344	3.4	2.1	265
	352	3.5	2.6	686
9039	400	4.0	2.4	450
	352	3.5	2.9	943
9040	440	4.4	2.0	136
9041	456	4.6	1.7	ND
9069	496	5.0	2.6	480
Effluent	352	3.5	2.8	857
duplicate tests	272	2.7	3.3	1667
9014B	312	3.1	7.2	5226
9015B	280	2.8	2.5	750
9031B	376	3.8	2.2	316
9039B	352	3.5	4.2	2057
9040B	312	3.1	2.7	871
9041B	328	3.3	2.7	818
9069B	360	3.6	1.8	
Effluent B	408	4.1	3.6	1318

DATE OF SAMPLE: OCT. 22, 1984

Station #	Total cell count (X106	Biomass (mg)	Microtox IC50 (TU)	TUs1/g
Effluent	328	3.3	2.4, ND**	ND
"	1100 (250mL test volume)	11.0	13.9, ND**	
Effluent-B	976 (500mL test volume)	9.8	12.3, 4.5**	-

DATE OF SAMPLE: NOV. 14, 1984

Effluent	448	4.5	6.5	3133
Effluent-B	480	4.8	2.2	250

DATE OF SAMPLE: DEC. 3, 1984

Effluent	320 (1000mL test vol)	3.2	10.4, 8.5**	-
Effluent-B	311 (1000mL test vol)	3.1	2.0	-

CONTROLS USED FOR CALCULATION OF AVERAGE CONTROL TOXICITY

(100 and 200mL test volumes)

DATE	SAMPLE/PROJECT	TEST VOLUME MLS	TOTAL CELL COUNT ($X10^6$)	BIOMASS (mg)	MICROTOX IC50 T.U.
27/6/84	Monsanto	200	-	4mg*	ND
4/7/84	BASF	200	-	4mg*	ND
5/7/84	GULF	200	-	4mg*	ND
10/7/84	Pt. de Mtl	100	-	2mg*	1.1
12/7/84	"	100	-	2mg*	2.1
13/7/84	"	100	-	2mg*	2.2
17/7/84	Pt. de Mtl				
	Gulf - Goodrich	200	-	4mg*	2.3
19/7/84	Pt. de Mtl	200	-	4mg*	1.3
25/7/84	"	100	-	2mg*	1.6
1/8/84	"	200	-	4mg*	1.2
7/8/84	"	200	-	4mg*	2.0
14/8/84	"Emissaire"	200	-	4mg*	2.7
15/8/84	"	100	-	2mg*	1.6
16/8/84	"	200	-	4mg*	2.1
31/8/84	"	200	500	5.0	1.9
6/9/84	"	200	329	3.3	2.4
7/9/84	"	200	359	3.6	2.2
11/9/84	"	200	298	3.0	1.5
12/9/84	"	200	376	3.8	2.8

CONTROLS USED FOR CALCULATION OF AVERAGE CONTROL TOXICITY

(100 and 200mL test volumes)

DATE	SAMPLE/PROJECT	TEST VOLUME MLS	TOTAL CELL COUNT (X10^6)	BIOMASS (mg)	MICROTOX IC50 T.U.
13/9/84	"Emissaire"	200	531	5.3	1.7
14/9/84	"	200	400	4.0	1.3
19/9/84	"Dragage"	200	360	3.6*	1.5
26/9/84	"Emissaire"	200	616	6.2*	1.6
26/9/84	"	200	448	4.5*	1.3
21/9/84	"	200	296	3.0*	2.9
2/10/84	"	200	288	2.9	2.5
3/10/84	"	200	320	3.2	1.9
22/10/84	"	200	480	4.8	ND
13/11/84	"	200	432	4.3	3.0
20/11/84	"	200	480	4.8	1.1
NOT USED FOR AVERAGE:					
3/10/84	"Emissaire"	500	1300	13.0	7.1,4.7**
10/12/84	"	1000	320	3.2	6.8,6.0**
18/12/84	"	1000	544	5.4	2.7

* biomasses estimated from initial innoculum

**corrected for colour/highest concentration removed from calculation (see Table V)

APPENDICE IV

BIOACCUMULATION - RECOMMENDED PROTOCOL II

Based upon results of experiments during the refinement of the bioaccumulation test, the following protocol is recommended for future tests: Chlamydomonas variabilis as the algal species to be cultured in AAP medium and counted using the Coulter Counter Model. Stock cultures should be started 8 days before tests are to be performed, under the following conditions:

Innoculum:	20,000 cells/mL
Volume of culture:	1L, in 4L Erlenmeyer flask
Media:	1 X AAP; with 10% buffer solution
	KH_2PO_4 (0.05M) and NaOH (0.029M)
Agitation:	manually, twice a day.

In preparation for a bioaccumulation test (BT), the cultures are counted and sufficient algae are concentrated to ensure a test innoculum of 500 X 106 cell/L per test. Concentration of the culture is separated by centrifugation at 500g's for 10 mins. The pellets are then recombined and resuspended in b H_2O; centrifugation may be repeated to ensure that the innoculum volume is 5% of the BT volume (1L).

This innoculum is added to 900mls of sample, previously filtered (on 0.2u). 100mls of buffer (10% v/v) and the AAP nutrient are then added. The BT period is 48 hours, following which time the algae are recouperated by centrifugation (500g for 10 mins.), and concentrated to a volume of 10mls. (volume adjusted if necessary with b H_2O). Lysis of the cells is accomplished in a nitrogen pressure cell at 2200psi for 5 mins.

The lysed cells are recovered (including the portion remaining in the pressure cell, and centrifuged at 1500g for 10 mins. The pellet and supernatent are carefully separated; the pellet is then frozen at -20°C for later analysis, if necessary.

The supernatent is then divided into two: one half to be filtered, the other to be irradiated. Buffered H_2O is added to the sample intended for filtration to bring the volume to 5mls. Filtration is performed in steps, using prewashed filters: 5.0u, 0.45u and 0.2u; the filtrate is collected and tested as follows:

Total vol = 5mls:

1mL = TOC

1mL = IC50 Microtox

3mL = IC50 Algal (Microplate Test with Selenastrum capricornutum

The other 4mls of lysate supernatant is vigourously agitated to ensure air saturation, 1mL of 5% H_2O_2 is added to increase the volume to 5mls. It is then exposed to ultraviolet radiation for 16 hours.

Cooling of the sample is accomplised by means of a water bath. The oxidized sample is then filtered on a prewashed 0.2u filter and analyzed for TOC and toxicity:

Total volume = 5mls: 1mL = TOC

1mL = IC50 Microtox

3mL = IC50 Algae (Microplate test)

The frozen pellet samples will undergo analysis when a technique for their complete oxidation is developed.

BIOACCUMULATION PROTOCOL II

Preparation of innoculum by centrifugation (1000RPM for 10 mins)

Innoculation of 900mls sample (control = buffered dH_2O) + 100mls buffer (10% v/v) + 1 X AAP with 500 x 10^6 cells/L (5% total volume)

48 hours of incubation

Recuperation of algae by centrifugation (1000 RPM for 10 mins)

Lysis of cells in Nitrogen Pressure Cell (NPC) (2200 PSI for 5 mins)

Collect lysed cells

Centrifuge lysed cells (3000 RPM; 10 mins)

Separate supernatant and pellet (pellet frozen for later analysis)

Divide supernatant into 2 fractions: 1 for filtration, 1 for irradiation + H_2O_2 (see text)

Toxicity in Microtox, MAT; analysis of TOC.

APPENDICE V

BIOACCUMULATION: IRRADIATION EXPERIMENTS

The results of the sampling programme indicated the need for a test with greater sensitivity and reproducibility. This was attempted by the following methods:

1) Increasing the sample volume to 1L; thus increasing the amount of potentially accumulable substances
2) Increasing the biomass (time of incubation, algal innoculum); thus maximizing the biological potential to accumulate substances
3) Decreasing the toxicity of the control lysates
4) Increasing the sensitivity of the detecting organism.

Steps 1 and 2 were carried out concurrently both with the surface water samples and experimental solutions. The latter included: DDT, PCB (Aroclor 1254), 2,4 dichlorophenol, diazinon, lead, tar sands effluent, and a highly toxic sample obtained from the Ministère de l'Environnement - Québec. Exposure periods were increased for some cases to 96 hours at test volumes of 1L and total biomasses approaching 10X those of the original test. No significant increase in test sensitivity was observed, due to the increased toxicity and variation in the controls.

Steps 3 and 4 dictated a change in the accumulating organism Selenastrum capricornutum. Various cell lysis methods were re-tested, yet none had the success of sonification. Unfortunately, this technique

contaminated the lysate with titanium (unlike algal tests, the Microtox test is relatively insensitive to metals). A literature study revealed that another readily available alga - Chlamydomonas variabilis was easily lysed using a non-toxic pressure method. (the Nitrogen Pressure Cell - NPC). This technique would thus eliminate metallic interference as well as permitting the use of a more sensitive organism for detection of toxicity - Selenastrum capricornutum and the Microplate Algal Test (MAT). Chlamydomonas can also be cultured in the same media as Selenastrum and can be counted on a Coulter Counter.

The toxicity of chlorophyll and/or colour had been inferred from previous tests, where lysate toxicity on the Microtox became non-existant after a colour test. Unfortunately, this colour correction was non-linear with respect to concentration (data not shown) and thus become highly subjective. Previous attempts to reduce the effect of chlorophyll-colour had involved filtration through a 0.2u filter instead of 0.45u, yet it was believed that higher biomasses would possibly have a greater efficiency of chlorophyll removal (due to clogging of the filters).

To remove chlorophyll, two techniques were attempted. The first involved combustion of the intact concentrated algae at 550°C, followed by acid digestion of the ashed pellet. This was then neutralized, filtered and toxicity tests done to detect toxicity of any accumulated non-organic substances. This was not successful due to the extreme toxicity of salts formed during the neutralization step.

The second approach was to oxidize the concentrated lysate using ultra-violet light, then use the filtered sample for toxicity testing. These experiments were performed with Chlamydomonas and various exposures to UV light. Results are presented in Tables VI - IX, along with details of each experiment.

The initial experiments were designed to test the effect of irradiation on toxicity; a prefiltration step was incorporated (using GF/A filters) because the first oxidation of a non-filtered lysate was observed to be incomplete. Irradiation using a prefiltration step seemed promising - the samples changed colour overnight from a dark green to a whitish beige and were then easily filtered on 0.2u. However, total organic carbon (TOC) results revealed the relative inefficiency of the oxidation step (approximately 14% removal due to irradiation alone - see table VII).

A third series of irradiation experiments (Table VII) re-tested non-filtered lysate samples. This result reinforced the earlier conclusion regarding the inefficiency of irradiation. One possibility was a lack of a source of ozone to completely oxidize the highly organic lysate. Therefore, experiments with the addition of H_2O_2 were performed (results in Table VIII).

These results show the improvement in oxidation efficacy with the addition of H_2O_2. The ideal balance between oxidative efficiency and toxicity is obtained with 1% H_2O_2 concentration ($>$65% removal, toxicity of 42 TU on MAT); further developments may improve this substantially. Therefore, it is recommended that H_2O_2, (for a final concentration of 1%), be added to lysed algae to complete the oxidation during the irradiation step.

It is also suggested that irradiated lysates and non-irradiated lysates be submitted to mass spectrophotometry; this could reveal changes due to oxidation and sources of possible background toxicity. Further developments of this test format could be attempted if this information was obtained.

TABLE VI

EFFECTS OF UV EXPOSURE ON TOXICITY

7 samples from initial concentrate (3mg/mL), volume of 10mls

Prefiltration: lysate filtered through GF/A before irradiation

Irradiation : for 24 hours

Toxicity and TOC : sample filtered on 0.2u before analysis

LYSATE SAMPLE	REMARKS	MICROTOX (TU)	MAT	COT (ppm)
No irradiation I		2.6	4.0	-
No irradiation II		4.3	48*	150
Irradiation	No prefiltration	2.6	15.4	-
Irradiation I	Prefiltration	2.5	8	-
II	"	2.2	37*	320
III	"	2.9	21*	270
Irradiation	Lysate centrifuged before UV; supernatant only irradiated.	---	16*	350

*effects of storage (at 4°C) on lysate may have affected toxicity.

TABLE VII

EFFECTS OF UV EXPOSURE ON TOC (WITH PREFILTRATION)

#1 and #2 lysates from same initial concentrate (2mg/mL), 10mL volumes

Prefiltration on GF/A

SAMPLE	REMARKS	TOC(ppm)	MAT (TU)
LYSATE #1	before GF/A filtration	720	
	GF/A filtration, before irradiation	360	
	Irrad. 19 hours	280	
	Irrad. 22 hours	300	
	Irrad 24 hours	260	
	Irrad 24 hrs, filt 0.2u	180	4.4
LYSATE #2	GF/A filtration, before irradiation	350	
	Irrad. 16 hours	330	
	Irrad. 20 hours	290	
	Irrad. 22 hours	270	
	Irrad. 24 hours	250	
	Irrad. 24 hours filt. 0.2u	230	8.0

TOC: valves obtained from representative sample, diluted in dH_2O and preserved with 2 drops conc H_2SO_4

TABLE VIII

EFFECTS OF UV EXPOSURE ON TOC

4 lysate samples from same initial concentrate (2mg/mL)

No prefiltration

SAMPLE	REMARKS	TOC(ppm)	MAT (TU)
LYSATE #1	No irradiation	775	
	Irradiation 16 hours	620	
	Irrad. 20 hours	640	
	Irrad. 22 hours	640	
	Irrad. 24 hours	660	
	Irrad 24 hrs, filt 0.2u	270	3.8
LYSATE #2	No irradiation	825	
	Irrad. 16 hours	620	
	Irrad. 20 hours	730	
	Irrad. 22 hours	455	
	Irrad. 24 hours	730	
	Irrad. 24 hours filt. 0.2u	335	4.0
LYSATE #3 (lysate centrifuged, superatant collected and irradiated)	No irradiation	685	
	Irrad. 16 hours	550	
	Irrad. 20 hours	350	
	Irrad. 20 hours, filt. 0.2u	350	4.6
LYSATE #4	No irradiation, filt. 0.2u	---	3.6

TABLE IX

EFFECTS OF H_2O_2 ON OXIDATION

-Algal samples (Selenastrum capricornutum) from two algal concentrates (>2mg/mL) volumes of 10mls/sample.

-No cell lysis: cells were intact after H_2O_2/UV treatment

SAMPLE AND REMARKS	TOC(ppm)	Microtox (TU)	MAT (TU)
Concentrate # 1: no treatment	1100	-	-
Concentrate # 2: " "	1000	-	-
Concentrate # 3: (16 h at 4°C)	690	-	-
1% H_2O_2 in conc # 1; 16h. irrad	390		
" irrad + filt'd 0.2u	270	3	42
2% H_2O_2 in conc # 2; 16h. irrad	580		
" irrad + filt'd 0.2u	290	22	72
5% H_2O_2 in conc # 1, 16h. irrad	(600)*		
" irrad + filt'd 0.2u	440	4	142
1% H_2O_2 in dH_2O; no irrad -	200	-	
1% H_2O_2 in dH_2O; irrad 16 h. -	19	72	

*Reported value 1200ppm; possible error in dilution of sample before analysis would result in double amount of TOC.

APPENDICE VI

BIOACCUMULATION OF LEAD BY CHLAMYDOMONAS VARIABILIS

Four experiments were performed using Chlamydomonas as the accumulating organism and lead as the test substance. Lead was chosen because of its low detection level chemically and the reported IC50 for Selenastrum which was confirmed to be quite high (~500mg/L); these two factors permitted higher concentrations of lead in the media and precise chemical analyses of small volumes.

These experiments are described briefly on the following pages, except for experiments #3 and #4. Unfortunately, the Coulter Counter was not properly calibrated for Chlamydomonas in the first two experiments, thus overestimating the cell populations. This apparatus suffered a malfunction during the 3RD experiment and was unavailable for use. Thus, biomass estimates for these experiments are rough estimates only.

All algal tests with Chlamydomonas involved test volumes of 1L and cell lysis by the Nitrogen Pressure Cell, except for samples of "intact algae".

Experiment # 1

Conditions:

Volume:	1L
Exposure:	48 hrs
Media:	2 X AAP
Innoculum:	800 X 10^6C/L
Lead:	286ug/L
pH (T°):	7.5
pH (T48):	10.0

Methods:

Media from exposed culture was pressure filtered on 0.8u and given to chemistry. The algal concentrates, and initial innoculum, were also prepared by pressure filtration. Later observations indicated that this procedure caused significant cell lysis.

Results:

Sample	Mat (TU)
Lead-Exposure	2-4
Control	8-16

Conclusions:

Analysis of the filtered media indicated a concentration of 116ug/L in the exposed culture; thus 170ug of lead was either precipated or bioaccumulated. The toxicity results were not indicative of expressed toxicity of bioaccumulated lead, through antagonistic effects could be involved.

Experiment # 2

Conditions:

Volume:	1L
Exposure:	24 h.
Innoculum:	2000 x 10^6C/L
Lead:	550ug/L in triplicate.

Methods:

The innoculum was prepared by centrifugation at 1000rpm (500g); this method does not stress the algae. The cultures were innoculated and allowed to grow for 24 hours; following this the pH was adjusted directly (with 0.1N HCl) and lead was added. Adjustment of the pH in this manner may have overly stressed the algae.

Results: expressed as absolute concentrations of lead

CHEMICAL

Sample	ug Pb
Lead Innoculated at T=0h	550
Lead in Filtered (0.8u) Media	4.0 (average of 3 values)
Lead in Intact Algae (not lysed)	6.2
Lead in Algal supernatant (centrifuged lysate)	2.6
Lead in Algal pellet (cell walls)	4.0

The precipitation of lead due to increase in pH was possible, as final pHs were between 8 and 9; this may explain why there was little bioaccumulation of the metal.

TOXICITY

Sample	MAT (TU)
Lead	1 - 2
Control	16 - 32

Due to the high background of the control, it was decided for the following experiments to test irradiation of the lysate to eliminate this problem as well as liberating organically bound metal; the use of a buffer to maintain pH between 7 and 8 was also attempted.

Experiment # 3

Conditions:

Volume:	1L
Exposure:	72 h.
Media:	1 x AAP with and without 10% buffer
Innoculum:	200 x 10^6/L
Lead:	552ug/L
pH:	7.0 - 7.4
Irradiation:	lysate prefiltered on GF/A
	irradiated for 24 h.
	filtered on 0.2u for toxicity tests
Buffer:	10% v/v added of: KH_2PO_4 (0.05M)
	NaOH (0.029M)

Methods:

The innoculum was prepared by centrifugation of stock cultures (500g's). The experimental cultures included 3 controls (irradiated and buffer, non-irradiated and buffer, and buffer only) and 3 similar conditions for lead exposure. As the Coulter Counter was not functioning, the biomass was estimated to be 60-80mg at 72 hours, due to the brillant green colour of the cultures.

LEAD CONCENTRATIONS DETERMINED CHEMICALLY

Culture Fraction	Experiment			
	Buffered		Non-Buffered	
	Relative (ug/L)	Absolute (ug)	Relative (ug/L)	Absolute (ug)
Conc. at T-O	552	552	552	552
Intact Algae	4500	45.1	14 800	178
Lysate	2000	14.3	-	-
Particles	16 000	16.3	-	-
Media T-72	130	130	56	56

Culture	Toxicity	
	Microtox IC50 (TU)	MAT IC50 (TU)
Control, non-irradiated and buffer	1 - 2	4 - 8
Lead, non-irradiated and buffer	4.8	4 - 8
Control, irradiated and buffer	-	stimulation
Lead, irradiated and buffer	2.3	19

Discussion:

The effect of irradiation appeared to be to liberate the lead bound to organics (approx 2000ppm for lysate), and toxicity was expressed. However, the non buffered culture concentrated more lead (3 x in the intact algae). This may be due to lower toxicity of lead, and possibly lower accumulation of the metal, in the presence of phosphates (eg: buffered cultures).

Experiment # 4

Aim:

To verify the promising toxicity results of the previous experiment; to permit chemical analysis of lead in all culture fractions including filters.

Conditions:

Volume:	1L
Exposure:	48 hrs
Media:	1 X AAP
Innoculum:	320 X 10^6C/L
Lead:	500ug/L
pH°:	7.15
Buffer:	10% v/v
Irradiation:	See experiment # 3

Methods:

The experiment involved 6 cultures

Control, no irradiation

Lead, no irradiation

Control, irradiation

Lead, irradiation

Lead, chemical analysis I

Lead, chemical analysis II

Cells were counted at end of test, using the new Coulter Counter properly calibrated for Chlamydomonas variabilis.

Results:

Culture	pH	Total Cell Count ($X10^6$)	Biomass* (mg)
Control, no irradiation	7.3	325	32.5
Lead, no irradiation	7.3	404	40.4
Control, irradiation	7.3	365	36.5
Lead, irradiation	7.4	414	41.4
Lead, chemistry I	7.3	450	45.0
Lead, chemistry II	7.3	436	43.6

*Based on experimental determination of weight of Chlamydomonas variabilis: 100×10^6 cells = 10mg.

LEAD CONCENTRATIONS DETERMINED CHEMICALLY

(averages of duplicate samples)

Sample	Relative Concentration ug/L)	Absolute Concentration (ug)
Lead Added T-O	550	550
Total Lead T-48	340	340
Media, non filtered	327	327
Media, filtered	600	540
Filter (0.45u)	425	42.5
Intact Algae	10	-
Algal Lysate	10	-
GF/A Filter (average of 3 samples)	24	2.4

The culture fractions for chemical analysis were as follows:

a) total lead at 48 hours: algae and media (in duplicate)

b) media, non filtered (in duplicate); (supernatant of centrifuged cultures)

c) media, filtered 0.45u (in duplicate)

d) 0.45u filter (in duplicate)

e) lysate, filtered on GF/A filter

f) GF/A filter (in triplicate)

g) intact algae

Since the non-filtered media (327ug) should equal the filtered media plus the filter (540 + 42.5); it appears that there was some either variation in the analysis or contamination of the filtered media. There was no bioaccumulation in the algae, although cell growth was also low.

Toxicity

Sample	MAT (TU)
Control, no irradiation	6.1
Lead, no irradiation	33*
Control, irradiation	3.8
Lead, irradiation	5.3

*"plateau" effect of growth; the IC50 is difficult to ascertain due to antagonistic factors.

Discussion:

The chemical analysis revealed no accumulation of lead; this was confirmed somewhat in the toxicity results, especially for irradiation treatment. The fact that limited cell growth may have affected bioaccumulation is possible. It is recommended that only

buffer acclimated or pH 7.0 acclimated cultures be used; in order to eliminate algal shock due to rapid pH changes. As well, cultures in buffer normally grow more slowly (one half non-buffered cultures); it is thought that a period of 48 hours of exposure may allow sufficient growth for bioaccumulation to occur.

APPENDICE VII

VOLATILIZATION EXPERIMENTS

Various experiments were attempted to increase the availability of accumulated inorganic substances for detection on the Microtox and MAT. All experiments used Selenastrum capricornutum, 1 X AAP media, volumes of 1L and various metals. The general protocol is described schematically in Fig. 1. A preliminary experiment with addition of 25u/L of Cu and neutralization with strong acids had a toxicity of 8.4 TU on the Microtox; the control was 8.0. In all 2 other experiments were done, these are described briefly. The technique does not seem promising because of the tricky and time consuming process of neutralizing small volumes, and due to the fact that the resulting salts are extremely toxic yet tend to antogonize metal toxicity.

VOLATILIZATION EXPERIMENT # 1:

Conditions:

Volume: 1L

Exposure: 24 hours

Innoculum: 500 X 10^6 c/l

Copper: 25ug/L

Results: The ashed pellet was solubilized with 3mls of 18N HCl, then neutralized with 3mls of ION NaOH. Final neutralization to pH 7.0 was donw with 0.1N HCl and 0.1N NaOH.

SAMPLE	TOTAL CELL COUNT $X10^6$	DRY WEIGHT (mg)	MICROTOX IC50(TU)	MAT (TU)
Control	1700	150	8.0	16-32*
Copper	1660	17.9	8.4	250-500*

* visual confirmation

- a control using SQ H_2O was also done in same series of experiments, giving a Microtox IC50 of 7.9 TU (ie: effect of acid/base only).

The conclusion was that it may be possible to detect accumulated non-organic substances using this technique and the MAT.

Experiment #2

Conditions: (3 cultures with different metals)

Volume: 1L

Exposure: 48 hrs

Innoculum: 600 x 10^6 cells/L

Cadmium: 3ug/L

Copper: 35ug/L

Lead: 200ug/L

Results: - solubilization with 3mls of 1N H_2SO_4

- neutralization with 1.5mls of 2N NaOH

CELL COUNTS ($X10^6$)

Sample	24 hours	48 hours
Control	2520	2600
Copper	2380	3100
Cadmium	3190	4700
Lead	2320	2900

TOXICITY

Sample	Dry Wt (mg)	Microtox IC50 (TU)	MAT (TU)
Control	26	Stimulation	25
Copper	30	1	23
Cadmium	50	1	37
Lead	25	1	29

Conclusion: It was concluded that the control toxicity (background) may be due to toxicity of the salt formed during neutralization. Weaker acids and bases were used during the next experiment.

Experiment # 3

(Included a chemical analysis of fractions for Pb)

Conditions:

Volume: 1L

Exposure: 48 hours

Innoculum: 250 x 10^6 cells/L

Lead: 300ug/L (in triplicate)

pH_o: 6.0

Results: - no final cell count was done, due to malfunction of Coulter Counter.

- solubilization with 0.1 NHCl; neutralization with 0.1N NaOH or 0.1N KOH.

Final pH and Dry Weight

Sample	pH at 48 hours	Dry Weight (mg)
Control	9.4	31
Lead #1	9.4	29
Lead #2	9.5	35
Lead #3	9.6	27

LEAD CONCENTRATIONS DETERMINED CHEMICALLY

averages of triplicate cultures (Pb)

Sample	Relative Concentration	Absolute Concentration
Control Media	2ug/L	2ug/L
Lead Media	6ug/L	6ug/L
Control - Rinsed Container	2ug/L	0.2ug
Lead - Rinsed Container	2ug/L	-
Control - Algae	40ug/L	0.2ug
Lead - Algae	33ug/L	0.2ug

- chemical analyses on algae after volatilization, solubilization and neutralization

TOXICITY RESULTS

Sample	MAT (TU)
Lead #1 neut with 0.1N NaOH	12.5
Lead #2 neut with 0.1N KOH	29
Lead #3 neut with 0.1 KOH	37
Control neut with 0.1N KOH	37
NaCl (10% sol'n)	125-250
KCl (2%)	125-250

Conclusions: The chemical results show no bioaccumulation of lead, possibly due to two reasons:

1) pH increase over 48 hr test precipatated the metal; making it unavailable for uptake
2) the volatilization treatment itself caused the disappearance of the metal. The technique used for extraction of lead in fish liver is a more exacting, gentle one.

It is possible that the volatilization technique used is not suitable for extraction of inorganics. Therefore, this technique is not recommended unless the volatilization step resembles that used for extraction of metals from animal tissues.