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MICROBIOLOGICAL AND TOXICOLOGICAL STUDIES OF STREAMS

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MANAGEMENT PERSPECTIVE

This paper synthesizes, in terms that the lay public will understand, some of the processes that occur when a pollutant enters a stream or river. A series of studies are described to illustrate the points that:

1. bacterial movement and longevity from a single inoculum of fecal material can and does have a long distance and long-term effect;
2. microbial community structure, microbial densities and microbial activity rates in sediments can be used to provide valuable insights into the contaminant status of sediments and the stream.
3. trying to predict survival and recycling of microbial inputs into a water course for modeling aspects is a very complex proposition; and
4. the "battery of toxicant screening tests" approach can be a major factor in helping the understanding of pollutant/stream interactions. Also when an appropriate "battery of tests" is settled on, this approach will enable managers to make decisions on priority concerns and also observe the impact of their decisions by the data produced by the "battery of tests" technique.

PERSPECTIVE-GESTION

Dans cet article préparé à l'intention du profane, on décrit brièvement les phénomènes qui se produisent lorsqu'un polluant est introduit dans un cours d'eau. On examine une série de travaux pour illustrer les points suivants :

1. Le mouvement et la longévité des bactéries d'un unique inoculum de matières fécales déterminent des effets à longue distance et à long terme.

2. L'organisation, la densité et l'activité des populations microbiennes dans les sédiments peuvent nous donner une bonne idée du degré de pollution d'un cours d'eau.

3. Il est très compliqué de prévoir pour des fins de modélisation la durabilité et l'assimilation des produits microbiens dans les cours d'eau.

4. La "batterie de tests de dépistage des composés toxiques" pourrait être très utile dans l'étude des interactions entre les polluants et les composants des cours d'eau. Lorsqu'une batterie de ce genre aura été définie, les gestionnaires pourront décider quels problèmes sont les plus urgents et voir l'effet des mesures appliquées d'après les résultats des épreuves.

ABSTRACT

A summary of effects which occur when a pollutant enters a stream is presented. In order to try to understand the events which occur after a pollutant enters a stream or river a series of studies listed below were undertaken, and are described. Studies on the transport and survival of bacteria in fresh water systems; studies on the evalution of the role sediments play in the survival and multiplication of bacteria; and the development of a battery of tests which can be used to evaluate stream or river microbiological, biochemical and toxicant loadings so that areas of concern within a fluvial system can be prioritized.

RÉSUMÉ

On décrit brièvement les phénomènes qui se produisent lorsqu'un polluant est introduit dans un cours d'eau. Pour comprendre ce qui arrive, on a réalisé une série d'études sur les points suivants : déplacement et survie des bactéries en eau douce; évaluation du rôle des sédiments dans la survie et la multiplication des bactéries; élaboration d'une batterie de tests pour évaluer la charge microbiologique, biochimique et toxique d'un cours d'eau de façon à pouvoir déterminer dans quels secteurs du bassin hydrologique il faut agir en priorité.

Rivers and streams are dynamic living mobile masses of water which are continually being renewed, and similar to snow flakes no two samples of water are identical. Pollutants rarely enter rivers and streams at a constant rate, except from planned discharges such as sewage treatment plants and industrial discharges, and even here there is tremendous variation in the nature and concentration of the discharged contaminant.

A single micro or mega pollutant spill of chemical or fecal matter upon entering a stream or river undergoes a variety of events. As the pollutant slug enters and is transported downstream, many factors come into play such as, stream flow rate and volume, photodegradation, U.V. light sterilization, particulate matter size and concentration, adsorption or absorption of the pollutant to settling particles and the grazing activity of the microbiota.

Several scenarios can occur with single spills as well as continuous inputs. One event which occurs when the pollutant slug is a soluble chemical, is for the pollutant slug to be transported downstream, continually being diluted and sedimented until there is no further trace of the event in the water column. This implies once the pollutant slug has passed any point no record of its existence would be found in the water column one or two hours later. Another series of events which occur when the pollutant is a hydrophobic viscous chemical, e.g. oil, is for the pollutant to partially act as a floating slug of water, as well as, contaminating plant life, stream banks, debris and sediments as it flows downstream due to its

viscous nature. These contaminated areas serve as long-term sporadic inoculum sites. Since biodegradation of this type of contaminant is relatively slow, one time input of these chemicals have long term and great distance effects. In the third scenario, fecal material from cattle, pigs, wildlife, etc. are the pollutant. The fecal particles contaminated with bacteria can float with the whims of the currents, can settle into the immediate sediments for recycling and resuspension, can get entangled and disentangled with vegetation, shorelines and debris until finally the particles and bacteria settle into the sediment or are grazed upon as food.

The problems we face are that we do not know how far, how quickly and at what concentrations these pollution slugs move. When these pollution slugs become part of the sediment, we do not know how far this occurs from the input, at what concentrations it can be found, how long it will stay in the sediments before biodegradation takes place, or it is buried or it is recycled to the water column as a new product or the same chemical. We also do not know how long the various bacteria of fecal origin can survive in various water bodies and sediments.

In order to try to understand the events which occur when a pollutant enters a stream or river, our laboratory has undertaken the following studies:

1. Studies on the transport and survival of bacteria in fresh water systems;
2. Evaluation of the role sediments play in the survival and multiplication of bacteria, and

3. Development of a battery of tests which can be used to evaluate stream or river microbiological biochemical and toxicant loadings so that areas of concern within a fluvial system can be prioritized. In this article, descriptions and discussion of some of these studies are presented.

MICROBIAL TRANSPORT AND LONGEVITY STUDIES

Stream transport studies wee performed using the tracer organism Serratia marcescens (Dutka and Kwan, 1980). This organism produces a typical maroon red colony on nutrient agar and is a member of the family Enterobacteriaceae to which coliforms, fecal coliforms and Salmonella also belong.

Two stream studies were performed, one in the summer (July) and the other in the fall (October-November). A 200 l broth culture of S. marcescens was used to inoculate the stream, at chosen up stream sites. Gauze collection pads (Dutka, 1978) were suspended in the stream at various selected sites and were collected after 1, 2 and 7 day immersion periods. The five-fingered gauze pads were dismembered and deposited in various selected enrichment broths and, after incubation, loopfuls of the broth were transferred to tryptone soya agar plates containing an antibiotic mix. Growth of the red antibiotic resistant S. marcescens after a 48-72 hr incubation, indicated the positive retrieval of the organism from the stream.

In the summer study the tracer organisms were isolated after 3 days at a site 5.5 km from their input and they continued to be isolated here for a period of 8 days. It was estimated that it took the organism between 48 and 72 hours to travel 5.5 km. Stream flow velocities indicated that the tracer organisms should have reached the site in 29 hours.

In the October-November stuy, water movement rates indicated that only 34 h would be required for water masses to move through the 20 km distance length of this study. However, it took 7 days of travel time before S. marcescens was isolated at the last station. During this study the tracer organism was isolated for a period of 22 days at various sampling sites.

These data indicate that bacteria foreign to the stream environment can live for at least 22 days. The data also indicate that bacteria do not form a homogeneous system with a water body; instead they act as particulate matter which is sedimented or adsorbed to larger floating particles or caught in eddys, then released, or resuspended from the sediment into the main water flow to be carried further downstream. The data from this study suggest that no predetermined movement rate for water borne bacteria can be established.

Longevity of a bacterium is influenced by many factors, such as water temperature, sunlight (Dutka, 1984), grazer level, pH, oxygen level, organic content, toxicants, flow rate and physical environment. Since bacteria in nature can be free-floating, attached

to fine particles or embedded in a nutrient mass, it would be expected that survival times might vary. In an attempt to evaluate potentially different survival periods, two studies were undertaken to estimate the survival of free-floating, attached and buried bacteria. These studies were performed by placing membrane filter chambers (McFeeters and Stuart, 1972) in a fresh water pond and Burlington Bay (Dutka and Kwan, 1983).

In the first study, May to August, overnight broth cultures of Escherichia coli, Klebsiella pneumoniae, Salmonella thompson and Streptococcus faecalis were each inoculated (2 mL) into 200 mL of autoclaved Lake Ontario water. The inoculated lake water was dispensed in 100-mL portions into the membrane filter chambers; two chambers were prepared for each organism. The eight inoculated chambers were tested for baseline density levels and then placed on top of the sediment in a freshwater pond. After 21, 42 and 84 days, the chambers were retrieved, samples were collected from the chambers by means of sterile syringes, and the chambers were returned to the experimental sites. During sample collection the chambers were agitated in order to resuspend the bacteria that might have become attached to the inner chamber walls and membrane.

For the second study, November to May, the McFeeters and Stuart membrane filter chambers were modified by enlarging the capacity from 100 mL to 3000 mL. In attempting to mimic natural occurrences, it was believed important to increase chamber capacity to provide greater surface area (chamber walls and chopped agar) on which bacteria might

attach and would allow movement of the chopped agar so that surface bacteria might become surrounded with nutrient masses. The organisms E. coli, K. pneumoniae, S. marcescens, S. faecalis and S. thompson were evaluated during the winter longevity study. To each chamber was added 1 L tryptone soya broth augmented with 10 g beef extract and 1 L solidifed chopped tryptone soya agar augmented with 30 g beef extract. Each chamber was inoculated with approximately 150 mL of overnight broth culture of one of the organisms, and the remaining space in each chamber was filled with phosphate buffer. After the contents of each chamber were mixed and allowed to briefly acclimatize, baseline densiites for each organism were established. The chambers were then suspended in Burlington Bay, 5 metres above the bottom and 2.5 metres below the surface. The chambers were retrieved 162 days later and tested for microbial populations.

Results from the first summer study using lake water diluent, indicated that the four organisms, E. coli, K. pneumoniae, S. thompson and S. faecalis, were all able to survive the 84 day study period. Similar results were obtained from the winter study. All the test organisms, E. coli, K. pneumoniae, S. thompson, S. faecalis and S. marcescens, easily survived the 162 day immersion period.

This survival-longevity study, indicates that fecal and tracer organisms can easily survive an immersion period of 162 days. Thus any waterway, beach, lake or pond which has been subjected to fecal pollution, even once, can remain a source of pathogenic bacteria for a long time. Disturbances such as heavy rainstorms, swimmers or dredging activities could at any time recycle pathogenic bacteria

throughout the water column and eventaully into humans or animals directly or indirectly through breakdown in potable water treatment systems.

The bacterial movement and longevity study data, indicate that a single upstream inoculum of fecal material can and does have a long-term downstream effect. We firmly believe that the distance travelled by the indicator bacteria and the survival times displayed by the test organisms greatly underestimate natural conditions.

SEDIMENT-BACTERIAL RELATIONSHIPS

Our bacterial longevity studies were extended to evaluate the relationship between microbial community structures and microbial densities in sediments and contaminants in sediments. Over 100 surface sediment samples from the St. Lawrence River Basin were examined for various microbial physiological types and densities (Rao and Mudroch, 1986). These organisms were correlated to the concentrations of trace elements (Ni, Co, Cr, V, Cu, Pb, Zn, As, Fe, Mn and Ti) and nutrients (P and organic matter). Results of these investigations indicated that there was a relationship between microbial densities and trace element concentrations. The data also were suggestive that there was bacterial population inhibition in the sediments due to the presence of certain toxicants. Generally, we observed in these sediments high and low microbial density areas with the lower microbial density areas being associated with areas having high concentrations of trace elements.

Data from these studies were also supportive of the hypothesis that the availability of organic matter (nutrients) for complexation with trace metal contaminants is dependent on microbial density and activities. Thus microbial community structure, microbial density and microbial activity rates in sediments can be used to provide valuable insites into the contaminant status of sediments and the stream.

From these sediment-contaminant-microbial population studies we have been able to confirm that microbial survival and reproduction in sediments are variable from site to site. This variability is due in part to the contaminant status of the sediments. These data suggest that trying to predict the survival and recycling of microbial inputs into a water course for modeling aspects is a very complex proposition.

BATTERY OF TESTS APPROACH

In research investigations or routine monitoring of waters and sediments, a variety of techniques have been used to designate waterbodies or sediments that are degraded, or are being degraded or have potential to be degraded. The term degraded when used in the above manner covers a variety of conditions such as unacceptale levels of chemicals, unacceptable responses to bioassay tests, unacceptable levels of health indicator bacterial populations and pathogenic microorganisms, presence of algal blooms, or presence of aesthetically deterioriated waters due to floating debris.

In our laboratories we have undertaken a series of studies to evaluate the suitability of a variety of microbiological, biochemical and bioassay tests to become part of a "battery of test procedures" to identify degraded or degrading water bodies. The final "battery of tests" selected would be used nationally to prioritize specific water bodies and sediments for immediate remedial action or future investigations. The "battery of tests" approach will make it possible for chemists to decide which water and sediment samples will receive detailed chemical analyses and which samples can be omitted from the analysis routine. Also the "battery of tests" approach will make it possible to establish "hot spots", areas of immediate concern, which were not previously suspected due to inappropriate/inadequate or one-dimensional testing procedures. In the final selection of the "battery of tests" those tests which can be performed on samples stored (frozen or refrigerated) for a minimum period of 30-48 hours, would be given priority for selection.

The tests which are being evaluated for our battery of tests are: Water Column Tests: coliphage (Dutka et al., 1987), fecal coliform membrane filter test, using mFC agar, fecal streptococci membrane filter test using KF agar, the fecal sterols, coprostanol and cholesterol (Murtaugh and Bunch, 1967; Dutka et al., 1973), Microtox (Beckman, 1982), Spirillum volutans (Dutka, 1986), ATP-TOX System (Xu and Dutka, 1987), Algal-ATP (Dutka et al., 1986), SOS Chromotest (Fish et al., 1985), Daphnia magna (APHA, 1985) and Ceriodaphnia reticulata (Mount and Norberg, 1984).

Sediment and Sediment Extract Tests: fecal coliform, most probable number technique using A-1 Broth; Clostridium perfringens, most probable number technique with confirmation in litmus milk (Dutka et al., 1986), Microtox, ATP-TOX System, Spirillum volutans, Algal-ATP, SOS Chromotest, Daphnia magna and Ceriodaphnia reticulata.

The fecal coliform and fecal streptococci bacterial indicator tests used in these studies are the traditional tests used in North American water quality studies.

Coliphage: Coliphage are bacterial viruses (bacteriophage) which infect and replicate in lactose fermenting, Enterobacteriacae (coliforms and fecal coliforms). Since coliphage replicate only in coliform and fecal coliform organisms, the finding of coliphage in water samples also indicates the possible presence of these indicators and other pathogenic organisms including viruses. The coliphage procedure used in these studies is similar to that found in section 919C of the 16th edition APHA Standard Methods (1985). This procedure can theoretically detect one coliphage (1 coliphage in 100 mL of water sample) where water turbidity is not in excess of 25 NTU.

Clostridium perfringens. C. perfringens is probably the most widespread pathogenic anaerobic organism on earth and its distribution is considered to be ubiquitous (Bonde, 1963). The natural habitat of this organisms and the only place where it can form spores is in the colon of warm-blooded animals (Bonde, 1963). Its occurrence in nature is consequently dependent on the presence of fecal pollution. In our tests we examine for the spores of C. perfringens which which can survive for years in sediments.

In our assessment of the various microbiological procedures as future candidates for the "battery of tests" approach, we have found that the coliphage test and C. perfringens test can be done on samples, refrigerated for at least 36 hours without count variations. Thus these two tests are high on our priority list as bacterial candidates for the final "battery of tests".

Many studies (Murtaugh and Bunch, 1967; Dutka et al., 1973) have shown that certain fecal organic compounds, such as coprostanol and cholesterol have the potential of being used as indicators of recent fecal pollution. Coprostanol (5β-cholestan-3β-ol) is one of the major fecal sterols excreted by many higher animals and chickens. Cholesterol a precursor of coprostanol in the gut of mammals and chickens, is converted to coprostanol by chemical reduction and/or by anaerobic gram negative flora. As cholesterol is also found in eggs, milk, lard and wool grease, it is not as specific an indicator of fecal pollution as coprostanol.

Thus, the finding of coprostanol in water or sediments indicates contamination by excreta from either domestic wastes or runoff from pastures or barnyards. On the other hand the finding of cholesterol in water and sediments would be highly suggestive of fecal contamination. Water and sediments for these tests can be preserved and tested weeks after collection.

TOXICANT SCREENING

The following described toxicant screening tests can be performed on water samples and sediment extracts that have been preserved by refrigeration or frozen. Frozen samples can be examined at leisure 2 or 3 months after collection.

Sprillum volutans. The organism S. volutans is a large aquatic bacterium which is readily visible under low magnification. It has a fascicle of flagella at each end which, under normal conditions, form oriented revolving cones allowing the bacterium to move forward and reverse directions at will. During the reversing process the polar fascicles reorient simultaneously. To perform the test, S. volutans is added to a volume of the sample and the mobility of the organisms is observed with a microscope. If the sample is toxic but contains non-lethal levels of toxicants, S. volutans loses coordination, as both fascicles try to assume the head or tail orientation, thus preventing normal bacterial motion.

Microtox. Beckman Instruments, Inc. have devised a test for acute toxicants in water or sediment extracts, in which specialized strains of luminescent bacteria (Photobacterium phosphoreum) are used as the bioassay organism. This test is functional because the metabolism of the luminescent bacteria is influenced by low levels of toxicants and, occasionally stimulants. Any alteration of metabolism affects the intensity of the organism's light output. By sensing these changes in light output, the presence and relative concentration

of toxicants can be obtained by establishing the EC_{50} levels from graphed data: EC_{50} being that concentration of toxicant causing a 50% reduction in light from the baseline level.

Genotoxicity Test. The test consists of colorimetric assays of microbial enzymatic activities after incubating the bacterial tester strain (E. coli K12-PQ37) in the presence of various concentrations of water or sediment extract sample. An exponential growth phase culture of the E. coli is introduced into the cells of a microtitration plate containing samples and controls. After a two-hour incubation at 37°C, a chromogenic substrate is introduced, which lyses the bacteria and a colour develops after a short incubation. The intensity of the colour rection can be analyzed visually. For more precise analysis, the SOS Chromotest microplate can be read in a microtitration plate reader. The more intense the colour (blue) the greater the concentration of genotoxicant.

ATO-TOX System. The concentration of ATP per bacterial cell remains relatively constant and stable throughout all phases of growth (D'Eustachio and Johnson, 1968). Thus bacterial densities can be easily estimated by measuring the ATP content of the test system. When rapidly growing bacterial cells are exposed to toxicants, growth inhibition usually occurs. After several life cycles the toxic effect can be estimated by comparing sample cell growth to the control via ATP content. However, some toxicants not only inhibit bacterial growth but also affect the luciferase activity during ATP

determinations. Therefore, the observed light ouput reduction of the test system is the net result of the inhibition of both bacterial growth and luciferase (called "total inhibition of the ATP-TOX System). Luciferase activity inhibition can be determined by adding a standard ATP solution, as enzyme substrate, to the sample and to a distilled water control and measuring the light emission of the enzyme. In our studies, we use E. coli K-12 PQ37 strain, even though any bacterium or mixture of bacteria can be used in this technique.

Algal-ATP. The algal-ATP toxicant screening test is based on the inhibition of ATP production in cultures of the green algae Selenastrum capricorutum (Blaise et al., 1984). The ATP content of the stressed Selenastrum is measured by the procedure described in the Turner Luminescence Review 1983. The results are reported as a percentage of Relative Light Output (RLO) of the non-stressed controls which is 100%.

Daphnia magna. The Daphnia magna used in our tests is the largest of the Daphnia, often reaching 5 mm in size. The neonates (first-instar young) are 0.8 and 1.0 mm long and can be observed by eye. This stage is the one most commonly used for tolerance studies. Tests are performed on neonate Daphnia that have been released from the mothers brood chamber during the previous 24 hours. In the test, 10 neonates are used for each dilution of sample to be tested (APHA, 1985). The neomate Daphnia are observed at 1 hr, 4 hr, 24 hr and 48 hr, and the number of dead animals are recorded. A 24 hr or 48 hr EC_{50} value is then derived from the pattern of deaths observed.

Daphnia are less tolerant of toxic substances than are fish (Kemp et al., 1976).

Ceriodaphnia reticulata chronic toxicity Test. The Cladoceran, Ceriodaphnia reticulata is used to evaluate the chronic toxicity of a sample in our studies. In this test six beakers of approximately 30 mL volume are used for each sample dilution and control with one animal per beaker. Tests are performed with young animals that are as similar in age as possible (8 hr maximum). On the 3rd, 5th and 7th day of the test, the young are counted and discarded. During the test period the animals are fed daily. At the end of the test the number of young per original adult and the number of broods per adult are established against that obtained in the control sample. An average of 2.5 broods per adult in the controls has beeen used as the end point in some testing procedures (Mount and Norberg, 1984).

In order to compare water and sediment samples with each other and others collected nationally a point value scheme was devised, based on the results of each test procedure in order to allow for a ranking of samples from least concern to greatest concern. The format used to award points for specific data values is presented in Table 1. The point allocation scheme is biased and not scientifically defensible, but it reflects the authors' experience with various concentration levels of toxicant activity and health related bacteria in Canadian waters and sediments. The present rating scheme is a viable entity which will change with increased data accumulations and when greater experience is gained.

Samples with the most points are deemed to contain the greatest potential hazard to man and organisms found in the aquatic ecosystem. High toxicant levels may have reduced microbial levels/activity in some sediment samples; however, cause and effect relationships were not investigated. This is an area of future research.

SUMMARY

From our stream and river studies, we have learned that a single input of fecal material can pollute a water course for at least 20 km and 162 days. However, the movement of bacteria and particle bound bacteria in a water course appears to be so variable that at present this movement is difficult to predict, especially in slow moving water bodies. We believe more research is necessary on the movement of different sized and density particles with ab/adsorbed bacteria and chemicals, in order to estimate sedimentation rates and distribution patterns of bacteria and toxic chemicals in streams and rivers.

As our research studies have shown that sediment composition plays an important role in microbial survival and multiplication which in turn play an important role in the biodegradation and biotransformation processes, we believe more studies are required on the interrelationships between sediments (size and density) chemicals, bacteria and other stressing factors such as temperature, dissolved oxygen and sunlight.

The "battery of tests" approach will, when an appropriate "battery of tests" is established, be a major factor in helping the understanding of pollutant/stream/river interactions. Also, the "battery of tests" approach will enable managers to make decisions on priority concerns and observe the impact of their decisions by the new data produced by this battery of tests.

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Table 1. Point Awarding Scheme for Sample ranking, Based on Suspected Contained Hazards

E. coli fecal coliform fecal strepto-cocci sediment MPN 10g/100 mL water /100 mL MF	Coliphage water/100 mL	*Clostridium perfringens* sediment 10 g /100 mL MPN	Points	Coprostanol ppb	Genotoxicity Induction factor per 0.5 mL 10x water or 1:1 Milli Q water extract	Algal-ATP % Relative light Units per 200 µL 10x water or 1:1 Milli Q sediment extract	Points
<100	5-24	<25	1	<1.0	1.0-1.29	100-50	1
101-500	25-100	26-100	2	1-3	1.30-1.50	49-20	3
501-2500	101-250	101-500	3	3.1-5	1.51-2.0	19-1.0	5
2500-16000	251-1000	501-2500	4	5.1-7	2.1-3.0	.9-.1	7
16000-160000	1000-5000	2501-10000	7	7+	3.1+	.09-.01	10
160000+	5001+	10000+	10				

ATP-TOX System % Inhibition per mL sediment extract or 10x water	Microtox EC_{50}/g wet wt sediment or/mL	Points	Cholesterol ppb	Points	*Spirillum volutans* Water Sample	Points	Sediment Extract and 10x water	Points
1-30	.4+	1	<2.0	1	neg	0	neg	0
31-60	.40-.31	3	2.1-4	2	+	10	+	5
61-90	.30-.21	5	4.1-6	3				
91-99	.20-.11	7	6.1-8	4				
100	<.10	10	8.+	5				