03-155

Environment Canada Environnement Canada

Canada

CSO Flocs: An Electron-Optical Assessment of Nanoscale Structure and Microbial Health

BY:

G. Leppard and M. West

NWRI Contribution No: 03-155

ONAL WATER
RCH INSTITUTE

UT NATIONAL DE
CHE SUR LES EAUX

TD
226
N87
no.
03-155

CSO Flocs: An Electron-Optical Assessment of Nanoscale Structure and Microbial Health

Gary G. Leppard and **M. Marcia West,**

Urban Water Management Project, Aquatic Ecosystem Management Research Branch,
National Water Research Institute, Burlington, Ontario, Canada L7R 4A6

and

Electron Microscope Facility, Faculty of Health Sciences,
McMaster University, Hamilton, Ontario L8N 3Z5

NWRI Contribution Number: 03-155

CSO Flocs: An Electron-Optical Assessment of Nanoscale Structure and Microbial Health

Gary G. Leppard and **M. Marcia West,**

Abstract

Flocs from combined sewer overflow (CSO) events were examined by transmission electron microscopy. Nanoscale components of floc architecture and microbial ultrastructure were described using a correlative multi-method approach to sample preparation. A comparison with biota-rich flocs, from other aquatic environments described in the literature, showed the CSO flocs to be: (1) similar in fundamental architecture; (2) rich in microorganism diversity; and (3) highly enriched in bacteria which displayed ultrastructural correlates of good health. As is typical of biota-rich flocs, the bacteria of CSO flocs tended to be linked together into communities by nanoscale fibrils of extracellular polymeric substances. Despite the presence of heavy metals and viruses in their disturbed aquatic milieu, the CSO flocs did *not* show evidence of becoming a monoculture and they were definitely *not* microbial graveyards; these healthy features are inconsistent with some current notions of growth and survival in disturbed ecosystems. Noted are managerial implications of healthy organisms living at the bottom of a food web under stress.

Flocs dans les eaux de déversoirs d'orage : une évaluation optico-électronique de la structure à la nanoéchelle et de la santé des microorganismes

Gary G. Leppard et M. Marcia West,

Résumé

Des flocs provenant de déversoirs d'orage ont été examinés par microscopie électronique à transmission. Les constituants de l'architecture du floc à la nanoéchelle et l'ultrastructure des microorganismes ont été décrits à l'aide d'une approche corrélative de préparation des échantillons reposant sur plusieurs méthodes. Une comparaison avec des flocs riches en biote provenant d'autres milieux aquatiques décrits dans la littérature a montré que les flocs de déversoirs d'orage ont une architecture fondamentale similaire, qu'ils comportent une grande diversité de microorganismes, et qu'ils sont très riches en bactéries dont les caractéristiques ultrastructurales témoignent d'une excellente santé. Comme on l'observe normalement chez des flocs riches en biote, les bactéries des flocs de déversoirs d'orage ont tendance à être liées ensemble à la nanoéchelle au sein des communautés par des fibrilles de substances polymériques extracellulaires. Malgré la présence de métaux lourds et de virus dans leur milieu aquatique perturbé, les flocs de déversoirs d'orage *ne montrent aucun signe* de monoculture et *ne sont sûrement pas* des cimetières de microorganismes. La santé évidente des microorganismes dans ces flocs est incompatible avec les notions actuelles à propos de la croissance et de la survie dans des écosystèmes perturbés. On discute de certaines implications pour la gestion d'organismes sains vivant à la fin d'une chaîne alimentaire sous stress.

NWRI RESEARCH SUMMARY

Plain language title
What is the structure of a CSO floc, and what might electron-optical investigations tell us about the environmental activities of such flocs?

What is the problem and what do sicentists already know about it?
Flocs which enter receiving waters from combined sewer overflow events can transport contaminants. It is possible that these CSO flocs may possess unusual properties/activities which we should understand in our efforts to model contaminant transport in perturbed aquatic ecosystems. A question is on the table, "Do CSO flocs present anomalies which must be investigated to obtain a better understanding of floc impacts?" With regard to floc architecture, there have been no investigations using high technology to examine nanoscale structures which play roles in contaminant binding, floc settling and the modulation of virus predation. This paper is the first such investigation.

Why did NWRI do this study?
The Great Lakes Action Plan requested this investigation.

What were the results?
The CSO flocs: (1) were rich in the diversity of microorganisms present; (2) had an unusually high proportion of healthy bacterial cells, as diagnosed by ultrastructural criteria; (3) did not precipitate heavy metals from the bulk water phase; (4) were spatially separated from viral predators of bacteria by colloidal secretion products of bacteria; (5) were accumulators of nanoscale agglomerates of humic substance. Overall, the CSO flocs resembled those previously described, by electron-optical means, for wastewater treatment tanks. No anomalies were evident but there were surprises. The bacteria-rich flocs were taken from a physically perturbed ecosystem which contained toxic metals and natural bacterial predators, yet the bacteria within the flocs were remarkably free of cell damage! Also, our capacity to describe spatial associations between viruses and other floc components developed better than previously imagined, an achievement which will contribute to our upcoming efforts to enumerate aquatic viruses (including specific pollution indicators) within particulate transporters.

How will these results be used?
These results show that several phenomena not previously amenable to detailed analysis have changed status; they are now amenable to such analysis by novel technology which is available via an association of laboratories utilized jointly by NWRI and its "floc and colloid partners".

Who were our main partners in the study?
Great Lakes Action Plan - North Toronto Combined Sewer Overflow Treatment Facility - Wastewater Technology Centre, Burlington - Dept. of Pathology & Molecular Medicine, FHS, McMaster University, Hamilton

Sommaire des recherches de l'INRE

Titre en langage clair
Quelle est la structure d'un floc de déversoirs d'orage, et que nous révèlent les recherches optico-électroniques au sujet des activités de ces flocs dans l'environnement?

Quel est le problème et que savent les chercheurs à ce sujet?
Les flocs de déversoirs d'orage peuvent transporter des contaminants dans les eaux réceptrices. Il est possible que ces flocs possèdent des propriétés ou activités inhabituelles qu'il faudrait étudier dans les efforts pour modéliser le transport des contaminants dans les écosystèmes aquatiques perturbés. Il faut se poser une question : « Les flocs de déversoir d'orage présentent-ils des anomalies qu'il faudrait étudier pour mieux comprendre leur impact? » En ce qui concerne l'architecture de ces flocs, aucune étude à ce jour n'a fait appel à la haute technologie pour examiner les structures à la nanoéchelle qui jouent un rôle dans la liaison des contaminants, la sédimentation des flocs et la modulation de la prédation par des virus. Le présent document résume une telle étude.

Pourquoi l'INRE a-t-il effectué cette étude?
Cette étude a été effectuée dans le cadre du Plan d'action des Grands Lacs.

Quels sont les résultats?
Les flocs de déversoirs d'orage sont riches en microorganismes divers; ils présentent une proportion de cellules bactériennes saines inabituellement élevée, comme en témoigent les critères ultrastructuraux; ils ne précipitent pas les métaux lourds présents dans l'eau qui les entoure; ils sont séparés des prédateurs viraux des bactéries par des produits de sécrétion de nature colloïdale que ces dernières sécrètent; et ils accumulent des agglomérats de substances humiques à la nanoéchelle. Dans l'ensemble, les flocs de déversoirs d'orage ressemblent à ceux des réservoirs de traitement des eaux usées qui ont été décrits précédemment par des méthodes optico-électroniques. Aucune anomalie n'a été observée, mais des faits surprenants ont été constatés. Les flocs riches en bactéries ont été prélevés dans un écosystème physiquement perturbé renfermant des métaux toxiques et des prédateurs naturels des bactéries, mais les bactéries présentes dans ces flocs étaient remarquablement exemptes de dommages cellulaires! Il a également été plus facile que prévu de décrire les associations spatiales entre les virus et d'autres constituants de ces flocs. Ce qui qui facilitera les efforts ultérieurs dans le dénombrement des virus aquatiques (y compris certains indicateurs de pollution) au sein des transporteurs particulaires.

Comment ces résultats seront-ils utilisés?
Ces résultats montrent que plusieurs phénomènes auparavant impossibles à analyser de façon détaillée le sont maintenant grâce à la nouvelle technologie dont disposent des laboratoires auxquels ont accès l'INRE et ses partenaires qui étudient les flocs et les colloïdes.

Quels étaient nos principaux partenaires dans cette étude?
Plan d'action des Grands Lacs – Usine de traitement des eaux de déversoirs d'orage du nord de Toronto – Centre technique des eaux usées, Burlington – Département de pathologie et de médecine moléculaire, Faculté des sciences de la santé, McMaster University, Hamilton.

Introduction

This research examines CSO (combined sewer overflow) flocs for the first time using high-resolution, minimally-perturbing, electron-optical methods (life-like preservation, ultrathin sectioning, transmission electron microscopy) in conjunction with energy dispersive spectroscopical analyses of individually selected floc components. To apply best management practices to CSO problems, we must address knowledge gaps which constrain our understanding of exactly what is being transported during an overflow event (Marsalek & Kok, 1997; Ellis & Marsalek, 1996). This paper uses novel nanoscale technology to address our understanding of transported flocs and their microbial and contaminant burdens.

Correlative analytical electron-optical technology (Leppard, 1992) has recently been developed for examining floc structure to an extent not previously thought possible; it is centred around transmission electron microscopy (TEM). Many nanoscale examinations conducted thus far have contributed to an improved understanding of flocs, not only of their ultrastructure but also of structure/activity relationships (Liao et al., 2002, 2001, 2000; Liss et al., 2002, 1996; Webb et al., 2000; Finlayson et al., 1998; Leppard, 1999; Leppard et al., 1998; Droppo et al., 1997; Heissenberger et al., 1996a). For many diverse aquatic ecosystems (fresh waters, marine waters and engineered water treatment systems), the new technology is applicable to:

(1) visualizing the diversity and structural integrity of floc microorganisms;

(2) characterizing the individual colloid species which make up the floc matrix (with a focus on
extracellular polymeric substances, or EPS);

(3) describing the three-dimensional associations of microbes, EPS colloids and other (e.g., mineral) colloids which constitute the floc architecture;

(4) ascertaining contaminant associations with matrix and microbial components of a floc. Such potentially revealing investigations can be done currently at (near molecular) resolution limits as fine as 0.001 to 0.003 micrometres, depending on the specific technique of choice for visualizing ultrastructure preserved in a life-like or natural-state manner. There is a growing list of complementary and tunable techniques for application to diverse waters (Leppard & Arsenault, 2003; Leppard & Buffle, 1998; Lienemann et al., 1998; Leppard et al., 1996; Liss et al. 1996; Droppo et al., 1996a, 1996b). Additionally, the health status of individually selected bacteria, visualized within a floc, can be inferred from ultrastructural criteria with regard to the general categories of healthy, moribund or dead (Heissenberger et al., 1996b), including the presence of viruses (Leppard et al., 1997). Such high resolution information can extend light microscope observations on the microbial ecology of CSO flocs, including ecotoxicological observations.

The goals of this investigation are (a) to obtain new structural information which was not previously obtainable, (b) to examine toxic metal/biological structure relationships on a nanoscale basis, and (c) to ascertain new facts which are potentially useful for developing best management practices.

Methods

Sample Collection and Preparation for Subsequent Electron-Optical Investigation

Samples were collected in early November and late November of 2000 (by Dr. Quintin Rochfort; UWMP, EMRB, NWRI) at the North Toronto Combined Sewer Overflow Treatment Facility, using the TEM field kit described in and recommended by Liss et al. (1996). Relevant information on the treatment facility site and on the CSO research conducted there is found in Averill et al. (2001). In early November, water samples (containing suspended flocs) were taken from the inlet, the mid-tank and the outlet, after overflow events. After a late November event, three kinds of samples were examined. A raw water (untreated) sample was taken from the influent channel. One effluent sample was treated with a dose of 12mg/L of an experimental polymer; the other effluent sample was treated with 16 mg/L of the same experimental polymer. In each case, the active dose was 50 % of the applied dose. The experimental polymer was ZETAG 7873, a cationic coagulant distributed by CIBA.

In addition to the electron-optical microanalysis (below) for floc/metal associations, metal contamination of the CSO flocs was assessed by conventional wet chemistry means, using inductively coupled plasma mass spectrometry (ICP-MS, done at the Wastewater Technology Centre of Environment Canada in Burlington, Ontario).

To avoid potential colloid instability artifacts of sample storage (Leppard & Buffle, 1998), a modified version (Leppard et al., 1997) of the multi-method approach of Liss et al. (1996) was used to stabilize and prepare samples on site. Subsequent processing led to the production of ultrathin sections, for examination by TEM and by scanning/transmission electron microscopy coupled to energy dispersive spectroscopy (STEM coupled to EDS). Each of the three preparatory protocols for correlative TEM (outlined in Leppard et al., 1997) was initiated immediately at the sampling sites in Toronto, using the field kit applied to freshly-taken water samples rich in CSO flocs. The three protocols (#1 - direct embedding of sample in a hydrophilic resin, #2 - primary fixation in a buffered glutaraldehyde solution, and #3 - primary fixation in a buffered glutaraldehyde/ruthenium red solution) were used correlatively to overcome specific artifacts (extraction, dehydration, shrinkage) inherent to each one when used independently. The samples in capsules filled with hydrophilic resin were polymerized according to Leppard et al. (1997); the samples in the two chemical fixatives were subsequently washed, fixed in an osmium tetroxide-based secondary fixative, washed again, dehydrated by solvent exchange and then embedded in a hydrophobic resin (Leppard et al., 1997).

Sectioning of Embedded Samples and Documentation by TEM and STEM-EDS

All resin-embedded samples were sectioned with a diamond knife on a Leica Ultracut UCT

ultramicrotome. For ultrastructural observations on morphology, ultrathin sections of ca. 50 nm were produced, whereas ca. 100 nm ultrathin sections were produced for EDS (Chandler, 1977) to reveal the "heavy" element compositions of selected fine structures within sections. Those sections pre-selected for EDS analyses were stabilized by mounting them on formvar-covered grids and coating them with carbon (Liss et al., 1996). Sections (on grids) made from hydrophilic (Nanoplast) resin (Frosch and Westphal, 1989) were counterstained with 1% aqueous uranyl acetate for 3 hours, whereas sections (on grids) made from hydrophobic (epoxy) resin (Spurr, 1969) were counterstained with uranyl acetate (Lewis & Knight, 1977) followed by lead citrate (Reynolds, 1963). All sections were examined and documented by TEM using a JEOL JEM 1200 EX TEMSCAN scanning transmission electron microscope (STEM) operated at an accelerating voltage of 80 kV in transmission mode. For element compositions of selected ultrastructural entities, EDS was carried out using a Tracor Northern detector with IXRF Systems Inc. EDS2000 software. The same values of instrument parameters were employed for all analyses. In TEM mode, the practical resolution limit for visualizing nanoscale structures is 1 nm for Nanoplast-embedded samples and ca. 3 nm for epoxy-embedded samples.

Search Protocol, Representativity and Quantification

Each ultrathin section was searched initially, using both low (ca. 5,000x) and medium (ca. 10,000x) primary magnifications, to get an overview of the colloidal (living and non-living) and macromolecular structures present. The initial search allowed representative regions (specific areas on the viewing screen) to be identified, based on morphological criteria and experience. Representative areas were documented by photomicrography at relatively high primary magnifications (ca. 25,000 to 40,000x) and used to identify abundant structures (bacteria, other microbes, fibrillar extracellular polymeric substances, viruses, mineral colloids, organic debris, colloidal humic substances) and interesting sites for subsequent EDS microprobe examinations. To infer bacterial health from bacterial ultrastructural integrity, the criteria of Heissenberger et al. (1996) were used; quantification of the data on a "per bacterium" basis yielded estimates of the proportion of healthy bacteria per sample type.

The EDS protocol was set up to determine the abundances of all elements ($Z>10$) present in measurable amounts within a given ultrastructural entity, using a standardless analysis (Reid et al., 1998); the irradiation times were 4 minutes each. For the purposes of this research, absolute quantification of elements was not required; instead, we determined the "abundances" (Jackson et al., 1999) of limnologically-relevant elements (including anticipated contaminant metals) present in measurable amounts, and then investigated element associations in precisely located individual colloids. Colloids selected visually for EDS analysis of ultrathin sections were those containing or associated with nanoscale agglomerations made visible by native electron opacity. The minimum detectable mass fraction for the elements of interest tended to 0.1%. A constraint on our capacity to detect and interpret relative abundances in ultrathin sections (essentially the relative intensity of X-ray emission of each element from the same concentration, or "P" as defined by Russ (1972)) was addressed by considering the $K\alpha$ line at 80 kV, which revealed that the elements of interest were detectable to similar extents (within a factor of 2).

Results and Discussion

New Structural Information on CSO Flocs

i) bacterial health/integrity

Individual bacteria in photomicrographs (selected by cross-sectional or longisectional images; see Figures 1-8) were rated by the ultrastructural criteria of Heissenberger et al. (1996b) as follows:
1) healthy (all essential structural components intact)
2) unhealthy to moribund (at least one essential structure missing or perturbed)
3) dead (cell devoid of cytoplasm).

Based on ultrastructural analyses of 201 individual bacteria, we assess the health of the bacterial population to be as indicated in Table 1. In support of the concept of good health for the bacterial populations is the fact that food storage and mineral storage granules were abundant in the cytoplasm of many diverse cell types. References to normal ultrastructure (Costerton, 1979) and plasticity (Costerton, 1988) at various organization levels in bacterial cells are readily found in the microbiology literature, as are references to relationships between the bioavailability of energy, population growth and cell survival (Morita, 1988).

Table 1

healthy	66.2%
unhealthy, grading to moribund	23.9 %
dead	10.0%

According to a literature survey carried out by Heissenberger et al. (1996b), using results based on (optical microscope) staining techniques (Table 2), *active bacteria (sampled from a diverse selection of surface waters) made up certain fractions of the total bacterial population as shown below*, depending upon the characteristics of a particular aquatic ecosystem. As is evident, bacterial activity is likely a function of multiple environmental factors. The depth "m" refers to metres below surface.

Table 2

North Sea (high salinity)	0 - 25 m	2 - 5 %
Kiel Bight (Baltic Sea) (low salinity)	1 m	6 - 7 %
Westensee, Germany (fresh)	1 m	23 - 36 %
Chesapeake Bay (saline and nutrient rich)	8.5 m	56 - 61 %

A comparison between floc-associated bacteria and free-living bacteria in the Northern Adriatic Sea by Heissenberger et al. (1996b), using our current TEM technology, suggests that life as part of a floc appears beneficial to bacterial health, a result supported by many microbiological studies based on standard techniques (Alldredge & Silver, 1988) . The rating of 51.1% healthy (reported by Heissenberger et al., 1996b) in Table 3 is considered a high number by microbial ecologists; by comparison, the rating for our CSO flocs was 66.2%.

Table 3

Adriatic flocs (marine snow)	5 - 30 m	51.1 % of floc cells were active/healthy
Adriatic free-living bacteria	5 - 30 m	34.1 % of free cells were active/healthy

ii) extracellular polymeric substances (EPS)

The overall significance of EPS in aquatic ecosystems is that it is a multi-functional material (Leppard, 1997) providing advantageous habitat (Droppo et al., 1997), while serving as building blocks for aggregates which cover a range of more than seven orders of magnitude in terms of the diameter of a single EPS fibril (Leppard, 1999). For engineered aquatic systems, some of the properties conferred upon flocs by EPS may be manipulable (Liao et al., 2002; Liss, 2002); consequently, there is a potential for cost-effective manipulations which could move water treatment closer to the ideal of best management practices.

The contribution of EPS to CSO floc architecture is assessed as follows. For inlet flocs of the first sampling (early November, 2000), EPS fibrils were not abundant, although some bacteria had short fibrils (< 100 nm) projecting from their external wall, and microcolonies were enclosed in well developed fibrillar capsules. Matrix material was represented mainly by colloidal humic substances and colloidal debris; an overall impression suggested that aggregation at this stage might be primarily an electrochemical phenomenon, rather than a biologically driven process. The mid-tank samples of the early November sampling revealed two distinctive floc types in terms of architecture; there were flocs resembling those sampled at the inlet and flocs containing a well developed matrix of EPS fibrils (Fig. 1). The outlet samples resembled the mid-tank samples.

For the second sampling (late November, 2000), the CSO floc populations in the raw influent were dominated by well developed fibrillar matrices, suggesting that the microbial aggregation had been biologically driven. Floc populations labeled "16 mg/L" (coagulant dosage) resembled those of the mid-tank samples of the first sampling, as did those labeled "12 mg/L".

Comparing these Toronto CSO flocs with other flocs recently analyzed by our regional "NSERC Floc Group" (S.N. Liss, D.G. Allen, I.G. Droppo & G.G. Leppard), using similar multi-method electron-optical approaches (Liss et al., 2002, 1996; Liao et al., 2002; Droppo et al., 1996a), we conclude that the fibril-rich CSO flocs resemble activated sludge flocs used in the treatment of wastewaters.

iii) microbial diversity

Microbial diversity was considerable in terms of cell size, cell shape, growth habit and specific nanoscale substructures present. The CSO flocs clearly contained a complex community of organisms rather than a community tending towards monoculture status. Bacteria in the process of cell division were common. Prevalent organisms were: Gram-negative type prokaryotic cells, with and without fibrillar EPS; Gram-positive type prokaryotic cells; a variety of prokaryotic organism species, as judged in terms of internal structure, with or without internal storage granules contributing to structure; spores; cells with the growth habit of single individuals, as members of microcolonies, as component cells of filaments or as pleiomorphs; virus-like particles identified as bacteriophage (Fig. 2); fungi (Fig. 3); and protozoa. Additionally there were bacteria with colloidal mineral coatings as described in Jackson et al. (1999). The Gram status for the eubacteria was assessed by us in micrographs on the basis of cell wall ultrastructural format, according to the correlative criteria of Beveridge & Graham (1991). There were no prokaryotic cells with dispersed thylakoids or eukaryotic cells with chloroplasts, meaning that photosynthetic microbes (cyanobacteria and eukaryotic microalgae) were not detected.

For the first sampling (early November, 2000), some examples of microbial diversity are revealed by the following images: (a) inlet sample (Fig. 4); (b) mid-tank samples (Fig. 5); (c) outlet samples (Fig. 6). For the second sampling (the raw water and two treatments of late November, 2000), some examples of microbial diversity are revealed by Figures 3, 7 and 8.

Figures 5 and 7 reveal individual bacteria which have accumulated nanoscale colloids at their outer surface. Such an accumulation phenomenon has been implicated in modulation of the interactions of toxic solutes with aquatic microbiota. For the case of humic substance colloids, there may be both direct physiological effects on individual cells and indirect effects which alter the bioavailability of a limiting nutrient or a toxic solute (Campbell et al., 1997). The use of TEM to analyze the interactions between bacteria and nanoscale colloids is an area of science which currently enjoys an explosion of new technology and new ideas.

iv) viruses

Colloidal particles in the size range of viruses (usually tens of nanometres) are readily detected by TEM-based techniques (Doane & Anderson, 1987). In general, "virus-like" particles can be tentatively identified by their colloidal dimensions and limited number of shapes. However, only those viruses with a distinctive morphology (as is the case for the bacteriophages shown in Fig. 2) can be identified by morphological criteria alone; unless one finds them specifically attached to a bacterial cell wall (in predator mode), one cannot assume that all such particles are infective viruses. The viruses identified in this research on CSO flocs were all bacteriophages; such viruses have shown good potential application as indicators of pollution (Armon & Kott, 1996).

Floc Architecture in Relation to Environmental Variables and Treatment

The fundamental floc architecture (Droppo et al., 1997; Liss et al., 1996) was similar for all CSO samples, although the extent of development of the fibrillar matrix was not the same for all samples (being weakly developed for early November inlet samples). Specific changes induced by treatments were not evident; however, a more extensive examination, involving laser scanning confocal microscopy and directed towards the coagulant *per se*, might be more informative.

Toxic Metal/Biological Structure Relationships at the Nanoscale

Metal levels in individual CSO water samples were measured by ICP-MS in micrograms per litre (Table 4); for metal concentration, the first number in Table 4 is a simple average and the second number (bracketed) is a flow-proportioned average (courtesy of the WTC).

Table 4

Metal	Time of Sampling	Location at site	Concentration µg/L
Cr	early November	influent (n=17)	7.96 (8.29)
		effluent (n=17)	7.90 (7.82)
	late November	influent (n=8)	8.30 (8.99)
		effluent (n=5)	7.11 (6.92)
Cu	early November	influent (n=17)	56.9 (57.2)
		effluent (n=17)	38.5 (41.6)
	late November	influent (n=8)	82.2 (96.0)
		effluent (n=5)	51.3 (55.8)
Fe	early November	influent (n=17)	1390 (1550)
		effluent (n=17)	905 (911)
	late November	influent (n=8)	1940 (2350)
		effluent (n=5)	1442 (1570)
Pb	early November	influent (n=17)	28.6 (32.8)
		effluent (n=17)	31.3 (30.3)
	late November	influent (n=8)	26.6 (29.4)
		effluent (n=5)	23.8 (20.6)

Zn	early November	influent (n=17)	97.2 (102)
		effluent (n=17)	82.0 (87.1)
	late November	influent (n=8)	134 (155)
		effluent (n=5)	104 (110)

General aspects are described for samples prepared initially in glutaraldehyde or Nanoplast to minimize element contributions (to EDS spectra) from preparatory fluids. Nanoparticles of high electron-opacity (= rich in "heavy" elements) were visually selected at random in TEM mode for subsequent element abundance analysis by EDS (Jackson et al., 1999). From the 70 spectra (53 extracellular colloids, 17 intracellular colloids), the heaviest element detected was Fe. Other elements detected were K, P, Si and Al (plus one example of Ti). Thus, if toxic heavy metals were present in the flocs (or floc parts), they would have to be present at abundances of less than one part per thousand on a per colloid/nanoparticle basis. Either the individual flocs were not concentrating heavy metals in specific floc parts (instead the colloidal floc parts may have sorbed heavy metals in mono- or pauci-molecular layers at surfaces), or, heavy metals were not taken up appreciably by the flocs.

A check on these findings was run by collecting spectra from flocs fixed initially in the glutaraldehyde-ruthenium red fixative, a fixative which provides an optimal combination of differential contrast and preservation of small colloids/nanoparticles. From the 53 additional spectra (43 extracellular colloids, 10 intracellular colloids), the heaviest element detected was Fe. Other elements detected were K, P, Si and Al (plus sometimes Ca inside cells). P was commonly found in intracellular granules, likely as a component of polyphosphate, since the bacterial chromosome was excluded from microanalysis. In the late November sample of flocs from influent raw water, some dense areas of matrix EPS were enriched in iron, a potential sorbent for many other metals. Considering the major inorganic components, Si and Al when present tended to be associated in the same colloid, with that colloid generally having the morphology of a clay mineral (Beutelspacher & van der Marel, 1968). However, the results from 123 total spectra show that contaminant heavy metals were not deposited on visualizable nanostructures as metal-rich agglomerates (neither on organic polymer surfaces nor on iron oxide-rich coatings).

To generalize broadly, the three different preparatory techniques for microscopy, and the different sample manipulations prior to preparation for microscopy, gave essentially similar results with regard to an absence of visualizable toxic metal agglomerations.

New Facts to Assist Best Management Practices

The good health status of our CSO floc bacterial populations and the diversity of the overall floc microbial population were obvious. It is evident that contaminants and physical disturbances in water do not necessarily mean catastrophe, or even physiological difficulties, to the microbes within CSO flocs. This has implications for best management practices from the modeling point-of-view. A major implication of our electron-optical investigation is that, while CSO flocs might adversely affect the health of some organisms which live in downstream water, it is a mistake to

perceive the CSO flocs themselves to be either microbial graveyards or biosystems converting to monocultures. When a combination of good nutrition and diversity is present within a floc, there is likely to be a variety of mechanisms used by the various microbes in a community to adapt to toxicants, with some of the mechanisms leading to immobilized toxicant species having altered bioavailability and stability. Consequently the floc microbes could potentially become (for predators/scavengers higher in the food web) a food source which is accompanied by toxicants of unanticipated bioavailability.

A decade ago, a related research thrust attempted to examine the ultrastructure of picoplankton as a correlate of picoplankton activity, and subsequently as an indicator of aquatic ecosystem health/perturbation (Leppard & Munawar, 1992). This exploratory work was supported by both Environment Canada and Fisheries & Oceans Canada. However, at that time, the correlative microscopical technology was not sufficiently advanced for a sustained research program; this situation has changed dramatically for the better.

Summary

--- Bacterial health in CSO flocs, as assessed by the ultrastructural integrity of cells, was found to be high when compared to literature accounts of the health of aquatic floc bacteria in general.
--- CSO flocs are not microbial graveyards, nor do they show signs of becoming a monoculture.
--- Fibrillar EPS was usually the major component of the floc matrix, within which the microbes were embedded and by which the microbes were linked together into communities.
--- Microbial diversity was considerable, including bacterial diversity.
--- Virus-like particles were detectable and viruses of distinctive morphology could be identified; the bacteriophages present are pollution indicators.
--- Toxic metals taken up from the aquatic milieu were *not* concentrated as nanoscale agglomerates in specific ultrastructural units of a floc.
--- Evidence was found for accumulation of nanoscale colloids at the bacterium/bulk water interface, a phenomenon which can modulate the entry into a cell of contaminants bound to the external colloids.
--- This summary is based on 2 sampling periods (6 samples total) in one season at the same facility.

Acknowledgments

The authors acknowledge the essential field contribution of Dr. Quintin Rochfort, the guidance of Dr. Jiri Marsalek, the cooperation of the North Toronto Combined Sewer Overflow Treatment Facility and of the (Burlington) Wastewater Technology Centre, and the generous financial support of the Great Lakes Action Plan.

List of References

Alldredge, A.L. and M.W. Silver (1988). Characteristics, dynamics and significance of marine snow. Prog. Oceanogr. 20: 41-82.

Armon, R. and Y. Kott (1996). Bacteriophages as indicators of pollution. Crit. Revs. Environ. Sci. Technol. 26 (4): 299-335.

Averill, D., P. Chessie, D. Henry, S. Kok, J. Marsalek and P. Seto (2001). Field experience with chemically-aided settling of combined sewer overflows. Proc. NOVATECH - Innovative Technologies in Urban Drainage Conference, Lyon, France. pp. 237-244.

Beutelspacher, H. and H.W. van der Marel (1968). Atlas of Electron Microscopy of Clay Minerals and their Admixtures: A Picture Atlas. Elsevier, Amsterdam. pp. 1-333.

Beveridge, T.J. and L.L. Graham (1991). Surface layers of bacteria. Microbiol. Rev. 55: 684-705.

Campbell, P.G.C., M.R. Twiss and K.J. Wilkinson (1997). Accumulation of natural organic matter on the surfaces of living cells: implications for the interaction of toxic solutes with aquatic biota. Can. J. Fish. Aquat. Sci. 54: 2543-2554.

Chandler, J.A. (1977). X-Ray Microanalysis in the Electron Microscope. North-Holland, Amsterdam.

Costerton, J.W. (1988). Structure and plasticity at various organization levels in the bacterial cell. Can. J. Microbiol. 34: 513-521.

Costerton, J.W. (1979). The role of electron microscopy in the elucidation of bacterial structure and function. Ann. Rev. Microbiol. 459-479.

Doane, F.W. and N. Anderson (1987). Electron Microscopy in Diagnostic Virology: A Practical Guide and Atlas. Cambridge University Press, New York.

Droppo, I.G., G.G. Leppard, D.T. Flannigan and S.N. Liss. (1997). The freshwater floc: a functional relationship of water and organic and inorganic floc constituents affecting suspended sediment properties. Water, Air & Soil Pollut. 99: 43-53.

Droppo, I.G., D.T. Flannigan, G.G. Leppard, C. Jaskot and S.N. Liss (1996a). Floc stabilization for multiple microscopic techniques. Appl. Environ. Microbiol. 62: 3508-3515.

Droppo, I.G., D.T. Flannigan, G.G. Leppard and S.N. Liss (1996b). Microbial floc stabilization and preparation for structural analysis by correlative microscopy. Water Sci. Technol. 34 (5-6): 155-162.

Ellis, J.B. and J. Marsalek (1996). Overview of urban drainage: environmental impacts and concerns, means of mitigation and implementation policies. J. Hydraul. Res. 34: 723-731.

Finlayson, J.C., B. Liao, I.G. Droppo, G.G. Leppard and S.N. Liss (1998). The relationship between the structure of activated sludge flocs and the sorption of hydrophobic pollutants. Water Sci. Technol. 37 (4-5): 353-357.

Frosch, D. and C. Westphal (1989). Melamine resins and their application in electron microscopy.
Electron Microsc. Rev. 2: 231-255.

Heissenberger, A., G.G. Leppard and G.J. Herndl (1996a). Ultrastructure of marine snow: II. Microbiological considerations. Mar. Ecol. Prog. Ser. 135: 299-308.

Heissenberger, A., G.G. Leppard and G.J. Herndl (1996b). Relationship between the intracellular

integrity and the morphology of the capsular envelope in attached and free-living marine bacteria. Appl. Environ. Microbiol. 62: 4521-4528.

Jackson, T.A., M.M. West and G.G. Leppard (1999). Accumulation of heavy metals by individually analyzed bacterial cells and associated nonliving material in polluted lake sediments. Environ. Sci. Technol. 33: 3795-3801.

Leppard, G.G. & A.L. Arsenault (2003). Quantification of individual native biocolloids in natural waters: assessing indicators of aquatic events, by using transmission electron microscopy with a modified approach to image analysis. Arch. Hydrobiol. (in press)

Leppard, G.G. (1999). Structure/function/activity relationships in marine snow. Current understanding and suggested research thrusts. In: Annals of the National Institute of Health of Italy (Annali dell' Istituto Superiore di Sanita) Monographic Series, Special Issue (ed. by E. Funari, F. Azam, S. Fonda Umani and R. Pagnotta), 35 (3): 389-395.

Leppard, G.G. and J. Buffle (1998). Aquatic colloids and macromolecules: effects on analysis. In:
The Encyclopedia of Environmental Analysis and Remediation, Vol. 1, ed. by R. A. Meyers. John Wiley & Sons, New York. pp. 349-377.

Leppard, G.G., D.T. Flannigan, D. Mavrocordatos, C.H. Marvin, D.W. Bryant and B.E. McCarry (1998). Binding of polycyclic aromatic hydrocarbons by size classes of particulate in Hamilton Harbor water. Environ. Sci. Technol. 32: 3633-3639.

Leppard, G.G. (1997). Colloidal organic fibrils of acid polysaccharides in surface waters: electron-optical characteristics, activities and chemical estimates of abundance. Colloids Surf. A 120: 1-15.

Leppard, G.G., M.M. West, D.T. Flannigan, J. Carson and J.N.A. Lott (1997). A classification scheme for marine organic colloids in the Adriatic Sea: colloid speciation by transmission electron microscopy. Can. J. Fish. Aquat. Sci. 54: 2334-2349.

Leppard, G.G., A. Heissenberger and G.J. Herndl (1996). Ultrastructure of marine snow: I. Transmission electron microscopy methodology. Mar. Ecol. Prog. Ser. 135: 289-298.

Leppard, G.G. (1992). Size, morphology and composition of particulates in aquatic systems: solving speciation problems by correlative electron microscopy. Analyst 117: 595-603.

Leppard, G.G. and M. Munawar (1992). The ultrastructural indicators of aquatic ecosystem health. J. Aquat. Ecosystem Health 1: 309-317.

Lewis, P.R. and D.P. Knight (1977). Staining Methods for Sectioned Material. North-Holland, Amsterdam.

Liao, B.Q., D.G. Allen, G.G. Leppard, I.G. Droppo and S.N. Liss (2002). Interparticle interactions affecting the stability of sludge flocs. J. Colloid Interface Sci. 249: 372-380.

Liao, B.Q., D.G. Allen, I.G. Droppo, G.G. Leppard and S.N. Liss (2001). Surface properties of sludge and their role in bioflocculation and settleability. Water Res. 35: 339-350.

Liao, B.Q., D.G. Allen, I.G. Droppo, G.G. Leppard and S.N. Liss (2000). Bound water content of activated sludge and its relationship to solids retention time, floc structure, and surface properties. Water Environ. Res. 72: 722-730.

Lienemann, C.-P., A. Heissenberger, G.G. Leppard and D. Perret (1998). Optimal preparation of water samples for the examination of colloidal material by transmission electron microscopy.

Aquat. Microb. Ecol. 14: 205-213.
Liss, S.N. (2002). Microbial flocs/suspended biofilms. In: The Encyclopaedia of Environmental Microbiology, Vol. 4, ed. by G. Bitton. Wiley, New York. pp. 2000-2012.
Liss, S.N., B.Q. Liao, I.G. Droppo, D.G. Allen and G.G. Leppard (2002). Effect of solids retention time on floc structure. Water Sci. Technol. 46 (1-2): 431-438.

Liss, S.N., I.G. Droppo, D.T. Flannigan and G.G. Leppard (1996). Floc architecture in wastewater and natural riverine systems. Environ. Sci. Technol. 30: 680-686.
Marsalek, J. and S. Kok, Eds. (1997). Great Lakes 2000 Cleanup Fund Program: Stormwater Management and Control of Combined Sewer Overflows. Theme Issue. Water Qual. Res. J. Canada 32 (1): 1-226.
Morita, R.Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. Can. J. Microbiol. 34: 436-441.
Reid, D.A., H.C. Ducharme, M.M. West and J.N.A. Lott (1998). Iron-rich particles in embryos of seeds from family Pinaceae. Protoplasma 202: 122-133.
Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.
Russ, J.C. (1972). Obtaining quantitative X-ray analytical results from thin sections in the electron
microscope. In: Thin-Section Microanalysis, ed. by J.C. Russ and B.J. Panessa. EDAX Laboratories, Raleigh, N.C. pp. 115-132.
Spurr, A.R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43.
Webb, S.M., G.G. Leppard and J.-F. Gaillard (2000). Zinc speciation in a contaminated aquatic environment: characterization of environmental particles by analytical electron microscopy. Environ. Sci. Technol. 34: 1926-1933.

Figure Legends

NOTE - The Figures themselves can be presented only as hard copies since they consume more than 16 MEGs in Word 7.

Fig. 1. A variety of bacterial cells embedded in a porous matrix of fibrils. The extracellular fibrils appear as very fine (grey) nanoscale bridges between individual bacteria. Some EPS fibrils are also cross-connected to each other and to a variety of small colloids. The bacteria, fibrils and small colloids constitute the CSO floc, whose size is much more voluminous than that revealed by the (ultrathin section) view shown here.

Fig. 2. Viruses of bacteriophage type (predators of bacteria), associated with the outer edge of a CSO floc (a floc not in the plane of focus).

Fig. 3. A cell of a fungus (tens of micrometres in major diameter) surrounded by many bacterial cells, most of which have dimensions at the sub-micrometre level.

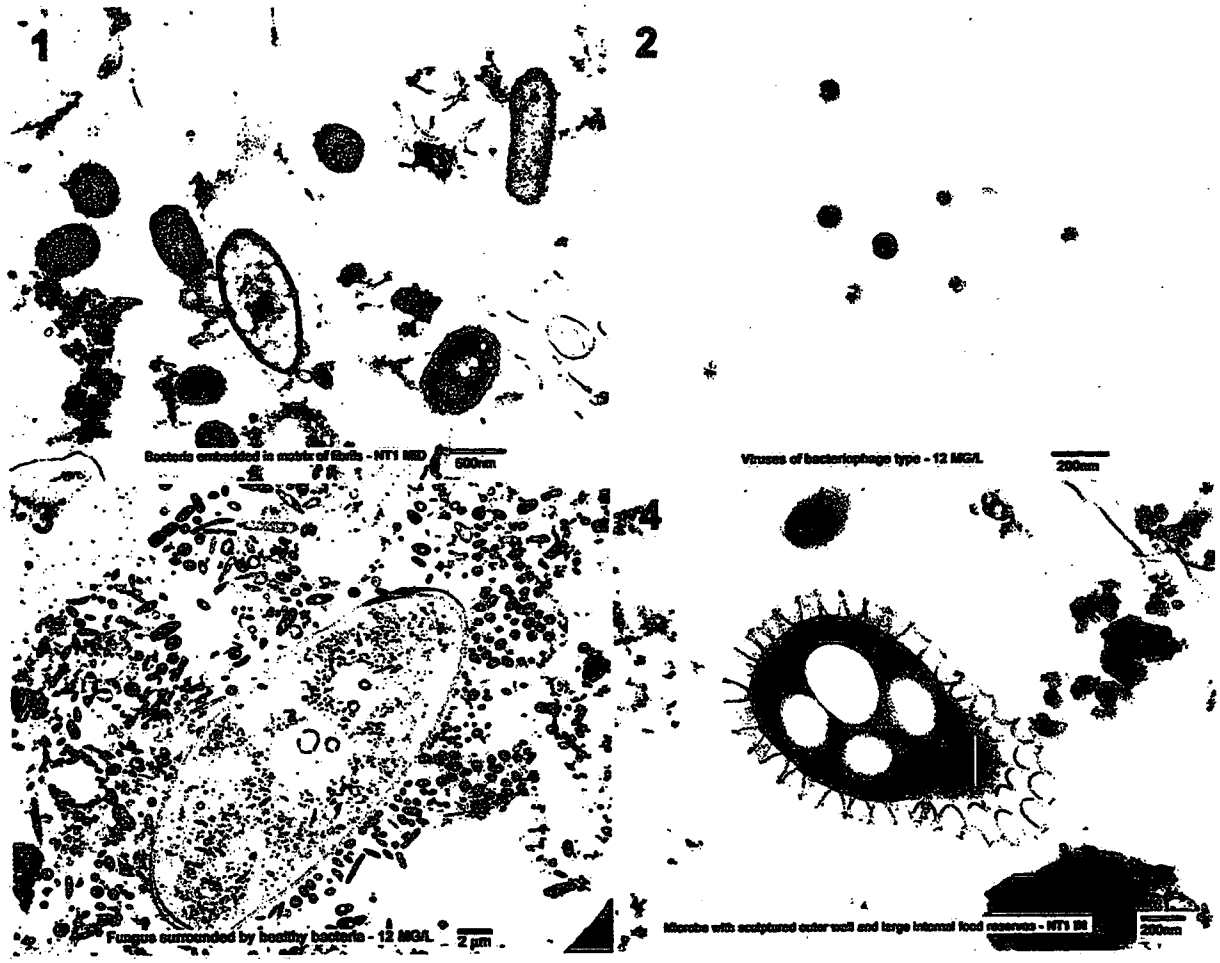
Fig. 4. Bacterial cell bearing a sculptured wall and large internal food granules. This morphologically unusual bacterium was found within an intra-floc pore, accompanied by some EPS fibrils and small colloids.

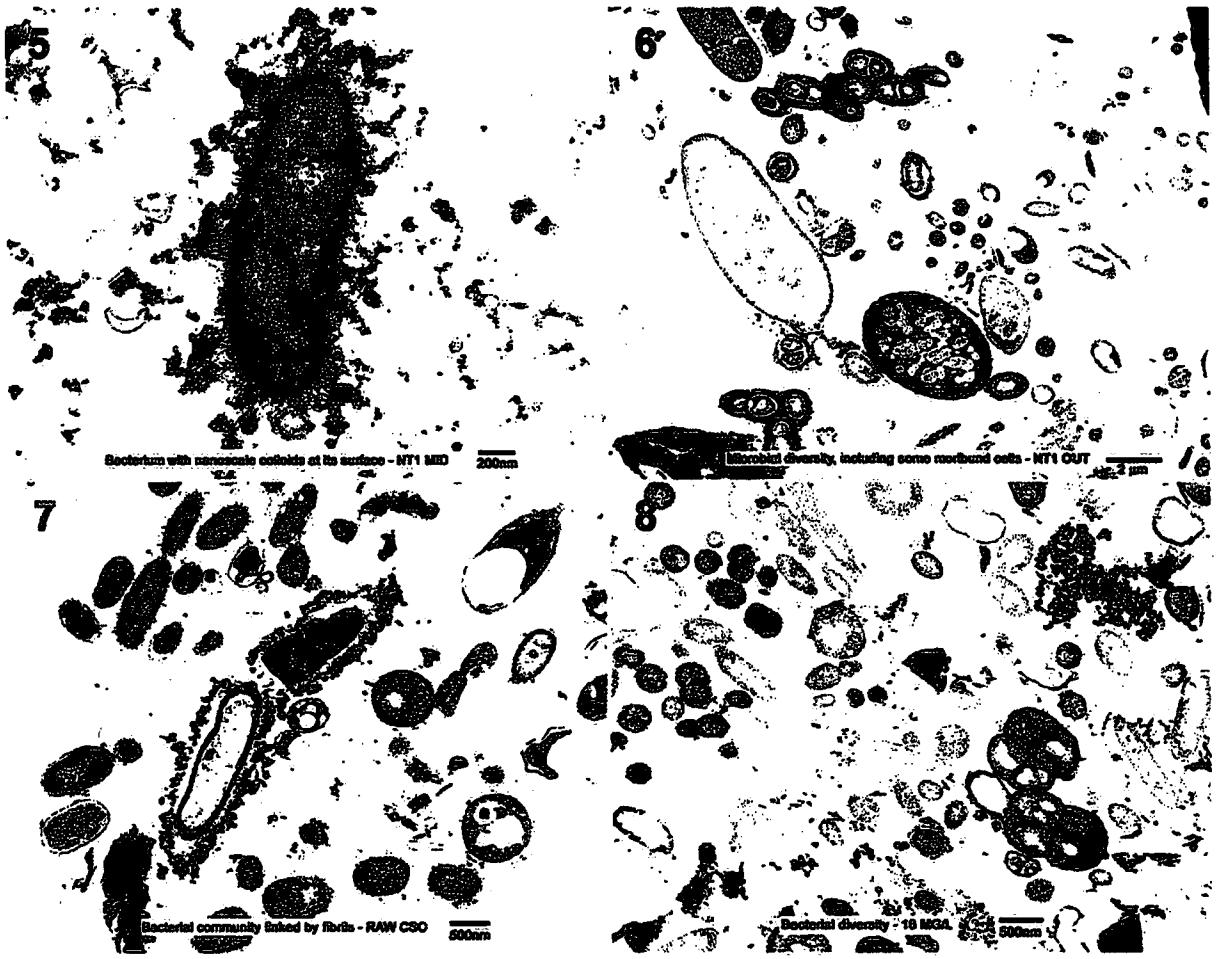
Fig. 5. Gram-negative bacterium found within an intra-floc pore. This high magnification view reveals fine details of intracellular structure and shows a major accumulation of nanoscale colloids at the bacterium/water interface. The Gram status of the cell wall was diagnosed using the ultrastructural format criteria of Beveridge and Graham (1991).

Fig. 6. Intra-floc microbes showing considerable morphological diversity. The large pale microbe at the left is moribund. The large dark microbe (bottom, centre) is full of granular food reserves (pale grey granules suspended in dark cytoplasm). Single small bacteria and colonial bacterial species are present, with and without internal food granules; they reveal a diversity of sizes, shapes and ultrastructure.

Fig. 7. More intra-floc bacterial diversity. Each of the two large central bacteria bears an external sheath which appears to be accumulating nanoscale colloids. Almost every cell has a healthy cytoplasm.

Fig. 8. More intra-floc bacterial diversity showing geometrically-regular colonies (groups of 4 cells each, lower right) and an apparent filamentous bacterium (extremely elongate cell, left of centre at top). Moribund cells are a minority.


1
2
Bacteria embedded in matrix of fibrils - NT1 IMD
500nm
Viruses of bacteriophage type - 12 MG/L
200nm
4
Fungus surrounded by healthy bacteria - 12 MG/L
2 µm
Microbe with sculptured outer wall and large internal food reserves - NT1 IM
200nm


5
Bacterium with nanoscale colloids at its surface - NT1 MID
200nm
6
Microbial diversity, including some moribund cells - NT1 OUT
2 µm
7
Bacterial community linked by fibrils - RAW CSO
500nm
Bacterial diversity - 10 MGA
500nm

Environment Canada Library, Burlington
3 9055 1018 1970 3

PRINTED IN CANADA
IMPRIMÉ AU CANADA
ON RECYCLED PAPER
SUR DU PAPIER RECYCLÉ

National Water Research Institute
Environment Canada
Canada Centre for Inland Waters
P.O. Box 5050
867 Lakeshore Road
Burlington, Ontario
L7R 4A6 Canada

National Hydrology Research Centre
11 Innovation Boulevard
Saskatoon, Saskatchewan
S7N 3H5 Canada

NATIONAL WATER RESEARCH INSTITUTE
INSTITUT NATIONAL DE RECHERCHE SUR LES EAUX

Institut national de recherche sur les eaux
Environnement Canada
Centre canadien des eaux intérieures
Case postale 5050
867, chemin Lakeshore
Burlington, Ontario
L7R 4A6 Canada

Centre national de recherche en hydrologie
11, boul. Innovation
Saskatoon, Saskatchewan
S7N 3H5 Canada

Environment Canada Environnement Canada

Canada