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EVALUATION DU RISQUE MICROBIOLOGIQUE D'ORIGINE HYDRIQUE : VALIDATION EPIDEMIOLOGIQUE DES FONCTIONS DOSE-REPONSE DU RISQUE VIRAL ET PARASITAIRE

Rapport d'étape, mars 2000

Convention d'étude du 24 août 1994
Agence de l'Eau Rhin-Meuse

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Plan :

- Travail réalisé depuis le dernier rapport d'étape
- Résultats provisoires concernant la qualité virologique de l'eau (projet d'article pour Water Science and Technology)
- Plan d'analyse statistique pour l'étude de relations entre qualité microbiologique et incidence de troubles digestifs aigus
- Calendrier prévisionnel pour la prochaine étape

• 1- Travail réalisé depuis le dernier rapport d'étape

Cette phase a été consacrée à trois activités :

- préparer une présentation des résultats concernant la qualité virologique des eaux dans les ressources et les réseaux de distribution ; cela a abouti à la présentation d'un projet d'article (non complètement finalisé), destiné à être présenté au Congrès de l'Association Mondiale de l'Eau à Paris, le 6 juillet (communication acceptée) et à être soumis pour publication dans la revue Water Science and Technology (projet joint à ce rapport d'étape).
- préparer le plan d'analyse statistique adapté au type de protocole mis en œuvre. Les données se présentent en effet comme une enquête longitudinale avec mesure répétée des observations sanitaires sur un panel de volontaires. Le phénomène statistique d'autocorrélation des variables sanitaires dans le temps doit donc être pris en compte (plan d'analyse présenté dans ce rapport d'étape).
- conduire une campagne complémentaire d'analyse de la qualité bactériologique de l'eau, à l'occasion de la période des pluies annoncées en février 2000. Ce travail complémentaire vise à augmenter la quantité de données disponibles sur la qualité de l'eau, et notamment lors des jours pluvieux, au cours desquels, pendant l'étude et son étape de faisabilité, les résultats microbiologiques avaient été les plus péjoratifs. Les résultats de cette campagne de mesure sont en cours d'exploitation et seront présentés ultérieurement; ils semblent montrer un niveau élevé de contamination virologique, mais non bactériologique, en distribution.

L'ensemble du travail a par ailleurs été présenté (L Gofti-Laroche) lors du séminaire organisé par le Ministère de l'Aménagement du Territoire et de l'Environnement à l'Institut Pasteur de Paris, le 27 janvier 2000.

2- Projet d'article sur les résultats de la surveillance de la qualité virologique de l'eau et de la morbidité incidente (pour Water Science and Technology)

"A NEW ANALYTICAL TOOL TO ASSESS HEALTH RISKS ASSOCIATED WITH THE VIROLOGICAL QUALITY OF DRINKING WATER (EMIRA STUDY)"

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Abstract

Introduction : Consumption of contaminated drinking water remains a source of gastro-enteritis outbreaks. Episodes have also occurred while drinking water complied with the microbial standards. Several studies suggest that enteric viruses may play a significant role. This work aims to assess the risks associated with virological quality of tap water using a molecular analytical tool manageable in a field survey.

Material and Method : The "EMIRA" study was carried out, between October 1998 and June 1999, in the French Alps. It combined a daily epidemiological follow-up of digestive morbidity among a panel of volunteers, and a microbiological surveillance of drinking water. Volunteers were recruited among communities supplied by 4 public water systems, with different degrees of raw water vulnerability (one "pristine" groundwater, one surface water, and two vulnerable ground watersheds); all complied with microbial (bacterial) quality criteria. Except in the first group (untreated), water was only disinfected by chlorine. Virological quality of water was assessed with monthly analyses in each group, using tangential ultrafiltration for concentration, and RT-PCR for detection of enterovirus, rotavirus and astrovirus. In addition to routine analyses, samples of tap water were stored (at 4°C) every

day by a local sentinel, and were analyzed in case an outbreak occurred. Bacterial analyses were also performed to control compliance of drinking water with microbial criteria.

Results : 712 cases of acute digestive conditions occurred to the 544 volunteers including 105 cases of diarrhoeic episodes and 46 gastro-enteritis. 24 raw water samples and 32 tap water samples were collected in routine; 12 additional tap water samples were analyzed during 4 alerts (but 3 showed non epidemic). 38% of raw water and 23% of tap water samples were positive for at least one virus marker. No enteric virus RNA was detected in the "control" group which was occasionally contaminated by faecal bacterial indicators; in the 3 other groups, the proportion of positive samples was respectively 11%, 37% and 53 %. 9 positive tap water samples out of 10 complied with bacterial criteria. A statistical analysis was performed to compare the observed incidence rates of observed digestive morbidity according to enteric viruses exposure.

In February 1999, an important outbreak occurred. Enterovirus and rotavirus RNA were detected in the 3 stored tap water samples, while no faecal bacteria was found. The bovine origin of the rotavirus RNA detected during this episode was evaluated by sequencing of the amplified fragments.

Conclusion : Enteric viruses markers were common in the tap water of communities with vulnerable raw water, despite absence of bacterial indicators. Tangential ultrafiltration coupled to RT-PCR allowed a good recovery, a simultaneous, fast and reproducible detection of the study viruses from environmental samples. This process appears as a promising analytical tool usable for virological water surveillance, inasmuch the corresponding know-how is transferred to the field professionals. The February outbreak gives the opportunity to investigate the link between an excess of digestive morbidity and viral molecular markers in tap water, whose health significance remains to be fully understood.

animals. It is a cause of diarrhea worldwide with reported detection rates between 5 and 9% among sporadic cases in young children. Recent evidence suggests that astroviruses are an important cause of acute non bacterial gastro-enteritis in children, adults and the elderly (Abad 97). Large outbreaks of astrovirus diarrhea are described with increasing frequency; it has been shown recently associated with diarrhea in HIV-infected patients. The role of astrovirus in episodes of gastro-enteritis in healthy young adults remains unclear, with 2 separate trials showing a very low rate of symptomatic infection among human volunteers (Belliot 97). The authors report that astrovirus can be a causative agent of a gastro-enteritis outbreak in healthy military recruits, but multiple potential enteric pathogens were also detected during the initial screening of stool samples. Advances in methods for detecting human astrovirus led to an increased understanding of their importance as a cause of gastro-enteritis in children and immunocompromised patients. Astrovirus is difficult to replicate in cell cultures, and needs molecular techniques for identification. Data on astrovirus survival in drinking water with chlorine disinfection scenarios suggest a better persistence at 4°C than 20°C; but in general, few data on the persistence of astrovirus in environmental samples exist at the present time (FX Abad, 1997).

Enterovirus genus (poliovirus, echovirus and coxsachievirus) is not considered as potentially involved in waterborne gastroenteritis (codex alimentarius). It could be responsible of various symptoms, but they are mostly considered as indicators of viral water quality rather than strict pathogens.

Infections associated with rotavirus or astrovirus among children occur after a short incubation (1 to 3 days). While enterovirus infections are most often asymptomatic or can be associated with various symptoms, a fever at 38°C is generally observed for rotavirus, and symptoms are observed during 1 to 2 weeks. (table 1) (Nicand 98).

MATERIAL AND METHOD

The EMIRA study was carried out, between October 1998 and June 1999, in the French Alps (Isère and Savoie departments, located in south-east France). One year before, a pilot study was performed to test the protocol feasibility. The EMIRA study combined a daily epidemiological follow-up of digestive morbidity among a panel of volunteers, and a microbiological surveillance of drinking water.

Panel follow-up

Volunteers have been recruited through the media, schools, town councils and community water systems files among communities supplied by 4 public water systems, especially chosen for their raw water vulnerability : one "pristine" groundwater located in a quarstic environment (1); two vulnerable ground watersheds : a quarstic watershed (2), and an unprotected watershed exposed to livestock and community sewage (3); and one surface water : a lake surrounded by human activities (4). All finished waters comply with the current European Union bacterial standards and, except in the first group which is untreated, water are disinfected by chlorine only. Each family had to complete a self administrated daily questionnaire whereby all health problems were to be registered. Each week day, one fifth of the panel families was interviewed by telephone, in the evening, in order to retrieve these data, thus collecting informations on the cases that occurred the same day and every day since the previous week call. This surveillance scheme allows a continuous description of digestive morbidity incidence, and outbreaks detection. An "alert" threshold was based on the pilot study data, defined as the occurrence of 2 cases of acute digestive conditions in the same community during 48 h. A case of acute digestive conditions (ADC) was defined as an episode of abdominal pain, nausea, vomiting and/or diarrhoea; a case of diarrhoeic episode (DE) was an episode of diarrhoea with at least another digestive condition; and a case of gastro-enteritis (GE) was an episode of diarrhoea with at least another objective sign as fever or vomiting.

microbial development or, conversely, of competitive inhibition), nor for protozoans (because the amount of water that must be sampled does not allow its storage by the local sentinel). Thus, bacterial water quality was assessed on the bottles sampled by the local sentinel the evening the outbreak was suspected (he was warned to do so immediately with 100 ml samples). Another aspect of EMIRA study dealt with the parasitical water quality which was assessed after filtration of a large quantity of water (100 l) through a polyethersulfone cartridge (Gelman® 1µm) at the sentinel home in the next morning. Besides microbial quality assessment during outbreaks, routine tap and raw water samples were collected by the technician every month. Because of logistic (accessibility) and financial reasons, analyses were carried out on tap water only in case of an alert, whereas they also included raw water samples for routine analyses.

Microbial analyses : Bacterial analyses were performed by the regional laboratory of water analyses to control compliance of drinking water to microbial quality regulation (absence of thermotolerant -"faecal"- coliform, of faecal streptococci, and of spores of sulfite-reducing clostridia). Also, protozoans (*Giardia and Cryptosporidium*) were analyzed by Immunofluorescence assay and enumerated by microscopy after Gelman® cartridge elution and clarification. Virological water quality was assessed using tangential ultrafiltration for concentration, and RT-PCR for viruses detection. We selected 3 categories of enteric viruses based on a review of the literature, and on technical feasibility : enterovirus, rotavirus and astrovirus. Water samples concentration and viruses detection followed the process set up in the laboratory of medical virology of Grenoble Hospital, and described in detail elsewhere (HS, WR). Tangential ultrafiltration was performed within 24 h following sampling (Minitan®, Millipore corporation, Bedford-MA-USA). Water samples of 4.5 ml were first concentrated to 15 ml and secondary to 0.5-1 ml. The concentrates were frozen at -80°C before molecular detection. RNA extraction was carried out after the second concentration with the Boehringer Kit High pure viral DNA. For enterovirus, RNA amplification borne on 400 bp in the 5' non-coding region, allowing detection of all human enterovirus. For rotavirus, the RT-PCR analysis was carried out using Beg9 and RTB primers to amplify a 392 bp fragment on the gene coding for the VP7 capside protein, allowing detection of animal and human rotavirus, and a semi-nested PCR with the RTA/RTB primers allowing to amplify a 341 bp. For astrovirus, RNA amplification was performed on 289 bp in the protease region (ORF1 a). Negative and positive control samples were included in all experiments. In our

RESULTS

The study population

The epidemiological follow-up of digestive morbidity involved 544 volunteers (176 households), with 27.9% of 0-14 years old subjects, distributed across the 4 water groups : 122 persons (40 households) in the control water group (n°1), 99 persons (32 households) in the quarstic water group (n°2), 100 persons (36 households) in the unprotected watershed group (n°3), and 223 persons (68 households) in the surface water group (n°4). As expected, in this kind of study based on a volunteer call, our panel is not representative of the general local population as to the socioeconomic status (SES); also, it comprises a greater fraction of children, a feature that was wanted (table 2).

Table 2 : Demographic characteristics of the study and the general populations

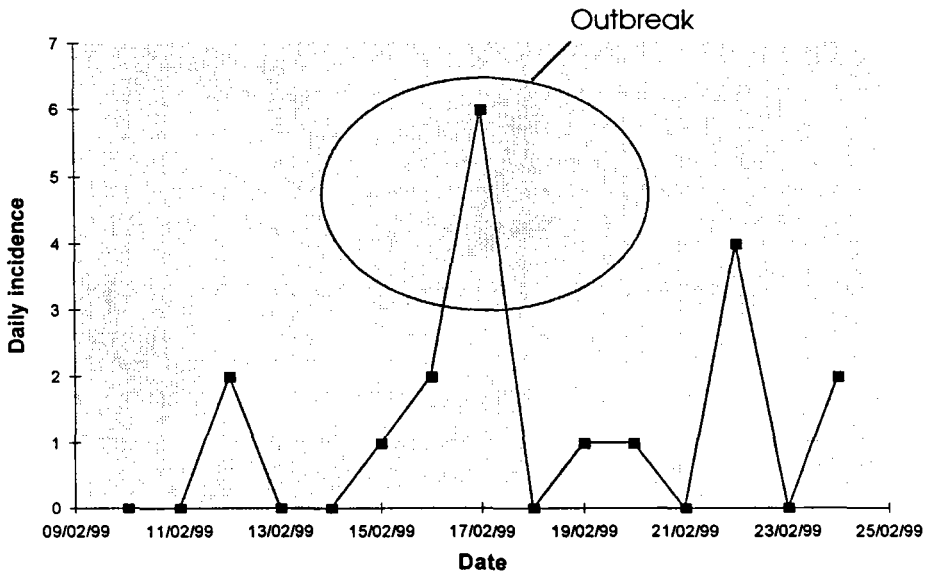
	Study population (n=497)	General population * (n=34 275)
Sex ratio	0.93	0.98
Age class	(%)	(%)
0-4 years	6.7	5.6
5-14 years	21.2	14.1
15-59 years	60.2	59.5
> 60 years	11.9	20.9
SES	(%)	(%)
Farmers	1.4	2.7
Craftsmen	7.5	8.7
White collars	13.6	8.2
Intermediate group	20.4	12.8
Employee	22.4	7.9
Blue collars	6.8	23.2
Retired	26.5	32.1
Others	1.4	4.4

** Source : Rhône-Alpes region general population statistics (INSEE)*

Digestive morbidity

The 20th of February 1999, the phone survey detected a significant increase of ADC in the third group around the 17th. The complete retrospective follow-up confirmed an outbreak. So, microbiological analyses were performed on tap water samples taken the 17th, 18th, 19th and 20th of February. This episode, along with daily incidence of ADC observed one week before and one week after the peak are shown in figure 3. 17 volunteers (19 cases) were involved in this outbreak. They do not differ from the 83 other volunteers in the same water group as to age (p=0.4), gender (p=0.9), SES (p=0.9), tap water intake (p=0.4), or tap water consumption outside their community (p=0.8). However, they show a greater background incidence rate of ADC than the other panelists : 6.7 person-year (49 incident cases/2667 person-days) versus 4.3 (124/10574) (p=0.007). The background incidence rate of ADC was defined as the incidence rate of ADC during the whole study, but during the epidemic period (i.e., out of 10 to 24/02/99). Given the little sample size, this difference also holds true for DE (1.0 vs 0.5 person-year) and GE (0.4 vs 0.1) (p=0.17 and p=0.07, respectively).

Figure 3 : Daily incidence of ADC in the third group around the outbreak peak (between the 10 and the 24th February 1999)



Water quality

8 routine sampling runs were performed between November 1998 and June 1999, and 4 epidemic alerts have been declared (one alert in each group), but 3 were not confirmed as true outbreaks. 24 raw water samples, and 32 tap water samples were collected in routine; 12 additional tap water samples were analyzed during the 4 suspected alerts.

of positive samples for viruses was respectively 11%, 37% and 53 %. Nine positive tap water samples out of ten complied with bacterial criteria. Enterovirus, rotavirus and astrovirus were respectively found in 13%, 15% and 12% of the samples (raw and tap water samples confounded). Table 5 presents virological results per community in the raw water (left column), and in tap water (right column). Raw water of the second group was rarely contaminated by viruses while raw water of the third and the fourth group were often contaminated (respectively 2/8 and 6/8). As to tap water, the first group did not show viral contamination. The second one, as on raw water, was rarely contaminated. The third group exhibited a high viral contamination during February and March 1999; in the fourth group, whose resource often showed astrovirus genetic material, tap water was routinely contaminated by enterovirus (but surprisingly not by astrovirus). Appendix 2 presents samples treatment, and shows the good reproducibility of the detection process.

The February outbreak in the third group : In February 1999, an outbreak occurred in the third group. Enterovirus and rotavirus RNA were detected in the 3 stored tap water samples, while no faecal bacteria was found. *Giardia* was also found in tap water the first day (10 cysts per 100 l). Further molecular investigations were not performed on enterovirus because it is not considered as potentially involved in acute digestive symptoms associated with drinking water consumption. Sequencing of the amplified fragments was performed to evaluate the origin (animal or human) of the rotavirus RNA detected during this episode, and 2 bovine strains were found (table 6).

Table 6 : Rotavirus sequencing results during and after the outbreak (appendix 1)

Date (sample number)		RT-PCR n°1	RT-PCR n°2	**Sequencing results** AA 21 26 39 41 45 47 69 86 103 104
17/02 (33)	sample*		+	
17/02 (34)	**sample****	+		**I M V T A T N V Q L**
18/02 (35)	**sample*****		+	**I T V T A T N V**
19/02 (36)	sample***	+	lack of material	
20/02 (37)	**sample*****	+	+	**M T I V T I D V Q L**
17/03 (43)	**sample******	+	+	**M T I V T I D V Q L**

* Tap water February routine sample (*Giardia* was also found)
** Raw water February routine sample
*** Tap water February epidemic sample
**** Raw water March routine sample
AA = Amino Acid

Association between digestive morbidity and enteric viruses exposure

The health indicator chosen for statistical analysis is the incidence of ADC; while less specific, it is more sensitive than the 2 others.

place where the bacteriological quality of tap water was not good (OR=2.7; p<0.02) regardless of the fact they drank water or not (HS, WR).

In developed countries, where there is a good level of hygiene and a low circulation of enteric viruses, the probability of immunizing contacts is lower and consequently, receptivity to infections may be higher than in developing countries (Nicand). Very few data are available on human immunity against the enteric viruses that were considered in our study. Some authors suggest that even if this immunity exists, it is very short, and limited to the infective genotype ( ).

Microbial water quality

Our results confirm that bacterial standards are not good predictors of potential viral risk through drinking water : enteric viruses markers were common in tap water of communities with vulnerable raw water, in spite of absence of bacterial indicators due to effective removal by chlorine disinfection. Presence of RNA viruses in water complying with bacterial standards has been reported previously (BGC). Since chlorine disinfection is less efficient to remove viruses, especially rotavirus, than bacteria, absence of faecal bacteria does not guaranty absence of viral markers. French water quality regulation requires a level of residual chlorine in tap water lower than 0,1 mg/l, a level clearly inadequate to remove viruses. Current microbial standards used as safety criteria for water may not always be indicative of viruses, therefore additional indicators of the virological quality of water are needed (HSWR). The World Health Organization underlined that because enterovirus, and the resting stages of *Cryptosporidium*, *Giardia* and other parasites, are more resistant to desinfection than *E. coli* and faecal streptococci, absence of the latter does not necessarily indicate absence of the former. Although, virological, epidemiological and risk analyses studies accumulate evidence in favour of this statement, direct virological water criteria still cannot be recommended, because of the complexity of virological analyses (WHO, 1993). Three options have been proposed to monitor virological quality of drinking water. The first consists of isolating the infectious enteric viruses in cell cultures. This is the ideal solution, but inapplicable for routine use because it is long, complicated and expensive. Furthermore, some enteric viruses of health significance do not (or poorly) multiply in cell culture (calicivirus, Norwalk like virus, VHA, rotavirus). The second option, currently used for water quality surveillance, is

techniques being rather quick and their cost steadily decreasing, it only allows to conclude that the analyzed medium is or was probably contaminated by viruses. The qualitative analysis (absence or presence) provides a partial information ; quantitative analysis is more relevant for health interpretation (Gantzer). The advent of molecular biology has prompted the development of procedures for the detection of fastidious enteric viruses ; however, molecular techniques fail to distinguish between infectious and non infectious particles, which may be of critical relevance in environmental virology. Molecular procedures are not adequate to monitor the presence of infectious viruses after disinfection (Abad, 97). Many authors suggest that viral genome detection might be considered as a viral contamination marker : detection of viral nucleic acid by RT-PCR is fast, cheap, very sensitive and routinely feasible. A drawback, however, is that viral genome in water is detectable a longer period of time (twice as long) as infectious viruses, particularly when particulate matter is present. Now, RNA persistence in water is 2 days maximum ; it is thus plausible that the detected RNA is encapsidated, these capsides not being able to induce cell infection (Gantzer 98). Moreover, chemical alteration of the nucleocapsides by chlorine also produces non infectious virions which, however, encapsidate a detectable RNA (Abad, 97). As a consequence, detection of viral genome in water cannot be considered, to date, as a perfect indicator of infectious virus.

Rotavirus sequencing

Sequencing of the amplified fragments (341 bp) was performed to evaluate the origin of the rotavirus RNA detected during February and March 1999. A similar bovine strain was found in the two first samples (17 and 18/02/99); another bovine strain was found in the two latter (20/02 and 17/03/99). The bovine origin of the detected rotavirus does not allow to conclude on its implication in the outbreak, yet, some authors suggest that interspecies crossing are possible with rotavirus (BGC). What can be said, however, is that rotavirus RNA occurrence in drinking water shows that viruses have been present in the drinking water supply during this episode, and that contamination by pathogen rotavirus remains possible. It should be noted that *Giardia* and enterovirus were also found in some February tap water samples. The codex alimentarius does not classify enteroviruses as waterborne pathogens responsible of gastro-enteritis; but *Giardia* could also be a candidate cause of this outbreak.

3- Plan d'analyse pour l'étude des relations entre la qualité microbiologique de l'eau et la morbidité incidente

L'objectif de l'étude consiste à modéliser l'incidence journalière des troubles digestifs aigus (variable binaire) en fonction de plusieurs prédicteurs, qualité et consommation et l'eau d'une part, et variables descriptives des personnes et des populations d'autre part. 543 individus ont été suivis au cours de l'étude longitudinale prospective d'octobre 1998 à juin 1999.

Les covariables sont :

✓ commune (variable qualitative) : les foyers de volontaires ont été recrutés dans 4 catégories de communes. Les critères de sélection des communes ont été : la plus ou moins grande vulnérabilité et le type de ressource en eau, et la présence ou non d'un traitement de désinfection chlorée. Sont ainsi définis les communes :

- Saint Laurent du Pont (Isère, 4083 habitants) : groupe 1
- Entre-deux-Guiers (Isère, 1544 habitants) et Les Echelles (Savoie, 1529 habitants) : groupe 2
- Drumettaz (Savoie, 1724 habitants) : groupe 3
- Aiguebelette regroupant 7 communes (totalisant 6000 habitants) : groupe 4.

✓ sexe (variable qualitative).

✓ age classes (variable qualitative) : les volontaires ont été regroupés en 4 classes d'âges :

- 0 - 4 ans
- 4 - 14 ans
- 14 - 59 ans
- 59 ans et plus.

✓ activité (variable qualitative) : 6 classes d'activités :

- Au foyer
- Scolaires ou étudiants
- Activité professionnelle
- Recherche d'emploi
- Retraité
- Autres.

✓ rob3 (variable quantitative) : consommation moyenne d'eau du robinet en litres par personne et par jour.

✓ bactério (variable qualitative) : des indicateurs bactériens sont recherchés pour vérifier la conformité réglementaire de l'eau en distribution (analyse sur 100ml). Ainsi, cette variable, combinant les résultats des analyses des divers indicateurs, a 2 classes :

- Conforme
- Non conforme

calibration et discrimination [5], seront utilisés pour mesurer la qualité de nos prédicteurs. Dans notre cas, le score de Brier (cas où Y est binaire) jugera la calibration c'est à dire la ressemblance entre y et $\hat{y}$ du modèle. La discrimination c'est à dire la capacité à différencier les individus se fera par les courbes ROC (Receiver Operating Characteristic Curve).

Par ailleurs, un des problèmes de notre étude est le nombre de données manquantes. La suppression de cases dans le tableau des covariables peut causer un biais et une augmentation de la variance. Des méthodes simples peuvent être utilisées pour remplacer les données manquantes, comme par exemple la médiane, la moyenne ou le mode mais celles-ci sont souvent biaisées et inefficaces quand les prédicteurs sont corrélés. Ainsi, une approche en incorporant les données manquantes dans l'analyse pourra être testée pour vérifier la puissance du modèle.

L'observation enseigne que l'incidence des troubles digestifs aigus a une forte temporalité. Ainsi, ce facteur sera pris en considération en prenant une variable temporelle binaire à caractère mensuel.

✓ Logiciel

L'analyse sera réalisée à l'aide du logiciel R version 1.0.0 sur station PC pour windows 95. R a été produit par Guido Masarotto et Brian Ripley. Il est disponible sur le site de la librairie statistique de la CMU à l'adresse internet suivante : http ://cran.r-project.org/bin/windows/windows-9x/base.

Le logiciel Oswald version 3.4 est une suite de fonctions conçues pour faciliter l'analyse des données longitudinales dans l'environnement Splus et donc dans R. Il est disponible à l'adresse internet suivante : http ://www.maths.lancs.ac.uk/Software/Oswald/.
Pour l'analyse statistique, ce logiciel sera utilisé et la méthodologie s'appuiera sur le livre de Diggle, P. J. ; Liang, K. Y. & Zeger, S. L. (1994) : Analysis of longitudinal data. Oxford University Press [6].

4- Prochaines étapes

Dans le prochain rapport d'étape, prévu au début de l'été, figureront :

- les résultats des enquêtes de consommation de l'eau (projet d'article)
- les résultats des analyses microbiologiques complémentaires effectuées en février, mises en relation avec les autres résultats d'analyse de l'eau de l'ensemble de l'étude (projet de 'short paper')
- les résultats de l'analyse statistique exposée plus haut, dans une première version de travail ; cette analyse sera poursuivie durant l'été et l'automne 2000.