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Devenir des virus entériques de l'Homme dans les eaux et les sols ; vers une comparaison de scénarios de rejets et de recyclages

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Résumé

Les eaux usées traitées d'origine domestique présentent généralement une charge non négligeable en virus entériques pathogènes de l'Homme, malgré leur traitement. Afin de pouvoir à terme comparer les risques sanitaires associés à différents modes de gestion de ces eaux usées, nous avons entrepris un travail sur (i) la prévision des quantités de virus entériques excrétés à partir des données épidémiologiques relatives aux gastroentérites aiguës, (ii) le devenir environnemental de ces virus lorsque les eaux usées sont rejetées en rivière, et (iii) le devenir de ces virus lorsqu'ils sont apportées au sol par irrigation. L'étude a porté sur un bassin de collecte des eaux usées de 240 000 habitants à proximité de Clermont-Ferrand, sur les rivières Artière et Allier et la nappe alluviale de l'Allier potentiellement contaminées par les rejets d'eau usée, et sur un sol du périmètre d'irrigation réutilisant ces eaux. Les concentrations en divers virus ont été suivies sur la même période dans les eaux usées brutes et traitées, dans les eaux douces de surface et souterraine. Nous avons proposé une méthode d'estimation du nombre journalier de nouveaux cas de gastroentérites aiguës d'étiologie virale en 2015-2016 à partir de données épidémiologiques et avons combiné ces estimations à un modèle d'excrétion virale pour évaluer les quantités de virus entériques arrivant à la station d'épuration. Le devenir des virus a été modélisé en tenant compte d'un abattement en station d'épuration et de dilutions-mélanges en rivières. Le devenir des virus apportés au sol par les eaux usées traitées réutilisées en irrigation agricole a été étudié sur un sol bien représenté dans le périmètre en utilisant un virus modèle. Ce devenir a été décrit par un modèle combinant transfert, immobilisation réversible et élimination, et en distinguant eau mobile et eau immobile comme virus libres et virus adsorbés sur des colloïdes en suspension. La méthode permettant de passer de l'épidémiologie à une excrétion de virus nous a permis de bien simuler les arrivées de virus à la station d'épuration avec un pic hivernal et l'impact prépondérant des norovirus GII sur les cas de gastroentérites virales. La simulation de l'abattement en station d'épuration et des phénomènes de dilutions-mélanges en rivière permet de simuler correctement la charge virale en aval du rejet d'eaux usées, mais leur devenir ultérieur reste mal caractérisé. Apporté au sol, le virus modèle était progressivement éliminé ou immobilisé de façon irréversible avec un abattement journalier de 0.38 $\log_{10}$. La fraction réversiblement immobilisée pouvait être estimée par une isotherme de Freundlich. L'ajout de Mg^{2+} a favorisé l'immobilisation du virus comme son adsorption sur des colloïdes dispersés dans l'eau mobile. Alors que les eaux usées stérilisées n'avaient pas d'effet majeur sur l'immobilisation du virus par rapport à une solution artificielle de sol en raison d'effets antagonistes des composés organiques et des cations minéraux, l'eau souterraine riche en Mg^{2+} favorisait l'immobilisation des virus. Un volet plante réalisé en marge de ce travail a montré l'impact d'irrigations sur les contaminations de surface et après internalisation *via* les racines. Complétée et améliorée, notre étude pourrait être couplée à une évaluation quantitative des risques viraux.

Mots clés : virus entérique, eaux usées, rejet, réutilisation, irrigation, devenir environnemental, scénarios, évaluation, modèles

Abstract

Urban treated wastewaters may be heavily contaminated by human pathogenic enteric viruses that cause acute gastroenteritis, despite their treatment. In order to compare health risks of different wastewater management scenarios, we investigated (i) how to predict virus shedding from epidemiological data on acute gastroenteritis, (ii) the environmental fate of these viruses when wastewaters are discharged into rivers, and (iii) the fate of these viruses when they are brought to the soil by irrigation. Our study focused on a wastewater collection basin of 240,000 inhabitants near Clermont-Ferrand, on the Artière and Allier Rivers and the Allier alluvial groundwater potentially contaminated by wastewater discharges, and on a soil in the irrigation perimeter reusing these wastewaters. Concentrations of various viruses were monitored over the same period in raw and treated wastewaters, as well as in surface and underground freshwaters. We proposed a method based on epidemiological data to estimate the daily number of new cases of acute gastroenteritis of viral etiology in 2015-2016; and we combined these estimates with a viral shedding model to estimate the quantities of enteric viruses arriving at the treatment plant. The fate of viruses has been simulated by taking into account the removal of viruses in the treatment plant and dilution-mixing in rivers. The fate of viruses brought to the soil by treated wastewater reused in agricultural irrigation was studied on a well-represented soil in the perimeter using a surrogate virus. Its fate has been described by a model combining transfer, reversible immobilization and removal; the model distinguished between mobile and immobile waters, as well as between free viruses and viruses adsorbed on colloids in suspension. The method for switching from epidemiology to virus shedding allowed us to accurately simulate virus inflows to the treatment plant, including a winter peak and the prominent role of norovirus GII in viral gastroenteritis cases. The simulation of virus removal in the treatment plant and subsequent dilution-mixing phenomena in rivers allow correctly simulating the viral load in the river downstream of the wastewater discharge, but their subsequent fate remains poorly characterized. When brought to the soil, the surrogate virus was progressively removed or irreversibly immobilized, according to a 0.38 $\log_{10}$ daily removal. The reversibly immobilized fraction could be estimated by a Freundlich isotherm. The addition of Mg^{2+} favored the immobilization of viruses, as well as their adsorption on colloids dispersed in mobile water. While sterilized wastewater had no major effect on virus immobilization compared to artificial soil solution due to the antagonistic effects of their organic compounds and mineral cations, groundwater rich in Mg^{2+} favored immobilization of viruses. An additional work, complementary to this PhD, showed the impact of irrigations on vegetable surface and internalized contaminations. After improvement, our study could be coupled with a quantitative viral risks assessment.

Key words: enteric virus, sewage, discharge, reuse, irrigation, environmental fate, scenarios, assessment, models

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Table des matières

RÉSUMÉ I

ABSTRACT II

REMERCIEMENTS III

TABLE DES MATIÈRES V

LISTE DES FIGURES VIII

LISTE DES PHOTOS XII

LISTE DES TABLEAUX XII

LISTE DES ABRÉVIATIONS XIII

INTRODUCTION GÉNÉRALE 1

CHAPITRE I. SYNTHÈSE BIBLIOGRAPHIQUE 5

1. LES EAUX USÉES ET LEUR RÉUTILISATION 6
2. LES VIRUS ENTÉRIQUES PATHOGÈNES DE L'HOMME 11
A. DESCRIPTION GÉNÉRALE DES VIRUS 11
B. LES VIRUS ENTÉRIQUES 15
3. DEVENIR ENVIRONNEMENTAL DES VIRUS (ÉTAT DES CONNAISSANCES) 20
A. DEVENIR DANS LES EAUX USÉES 20
B. DEVENIR DANS LES EAUX DOUCES ET SOUTERRAINES 24
C. DEVENIR DANS LE SOL 26

CHAPITRE II. PROPOSITION ET ÉVALUATION D'UN MODÈLE DE PRODUCTION DE VIRUS ENTÉRIQUES PATHOGÈNES DE L'HOMME ET DE LEUR DEVENIR EN STATION D'ÉPURATION, EN RIVIÈRES ET EN NAPPE D'ACCOMPAGNEMENT POUR SIMULER LES IMPACTS DE DIFFÉRENTS MODES DE GESTION DES EAUX USÉES 31

1. ABSTRACT: 33
2. INTRODUCTION: 35
3. MATERIALS AND METHODS 37
A. EXPERIMENTAL CONTEXT 37
B. EXPERIMENTAL DESIGN AND PROTOCOLS 39
C. WATER FLOW DATA 41

D. EPIDEMIOLOGIC DATA 41
E. VIRAL SHEDDING, TRANSPORT AND REMOVAL MODELLING 44
4. RESULTS AND DISCUSSION 46
A. ACUTE GASTROENTERITIS AND DRUG CONSUMPTION; RELATIONSHIP WITH VIRUS SHEDDING 46
B. VIRAL CONTAMINATIONS OF WASTEWATERS AND FRESHWATER BODIES; VIRUS FLOWS 51
C. FLOW OF VIRUSES; FITTING SIMULATIONS BASED ON EPIDEMIOLOGY AND SHEDDING TO ESTIMATES BASED ON MEASUREMENTS. 57
5. CONCLUSIONS 60

CHAPITRE III. PROPOSITION ET ÉVALUATION D'UN MODÈLE D'ÉLIMINATION ET D'IMMOBILISATION RÉVERSIBLE DES NOROVIRUS MURINS DANS UN PHAEOZEM CALCIQUE POUR DIVERSES CONDITIONS DE CONTAMINATION ET DE RINÇAGE 63

1. SUMMARY 66
2. INTRODUCTION 68
3. MATERIALS AND METHODS 71
A. VIRUS, SOIL AND ARTIFICIAL SOIL SOLUTION 71
B. EXPERIMENTAL DESIGN AND PROCEDURE 72
C. PROCESS MODELING, UNCERTAINTY ANALYSIS AND EXPERIMENTAL DATA ANALYSIS 75
4. RESULTS AND DISCUSSION 78
A. IMPACT OF TEMPERATURE, AGGREGATE SIZE, SOIL SATURATION PROCEDURE AND WATER FILTRATION ON VIRUS CONCENTRATIONS 78
B. REVERSIBLE AND IRREVERSIBLE IMMOBILIZATION IN SATURATED CONDITIONS OVER A WEEK 79
C. SOIL DRYING IMPACT ON THE REVERSIBILITY OF IMMOBILIZATIONS 83
D. THE IMPACT OF GEOCHEMISTRY ON IMMOBILIZATION AND SUBSEQUENT DESORPTION 84
5. CONCLUSIONS AND PERSPECTIVES 87

CHAPITRE IV. DISCUSSION GÉNÉRALE – CONCLUSIONS 89

1. RAPPEL DES OBJECTIFS ET FINALITÉS DE LA THÈSE : 90
2. DEVENIR DES VIRUS DANS UN CONTEXTE DE REJET : ACQUIS ET LIMITES DU TRAVAIL 90
3. DEVENIR DES VIRUS DANS LE SOL DANS UN CONTEXTE DE RÉUTILISATION D'EAU USÉE : ACQUIS ET LIMITES DU TRAVAIL 93
4. DÉFIS À ABORDER POUR ATTEINDRE LES FINALITÉS DU TRAVAIL 95

BIBLIOGRAPHIE 97

ANNEXE I. SURFACE AND INTERNALIZED CONTAMINATIONS OF GREEN ONIONS 1

ANNEXE II. VOMITING SYMPTOM OF ACUTE-GASTROENTERITIS ESTIMATED FROM EPIDEMIOLOGIC DATA CAN HELP TO ASSESS RIVER CONTAMINATION BY HUMAN PATHOGEN ENTERIC VIRUSES 1

ANNEXE III. SPATIAL CORRELATIONS IN DAILY ACUTE GASTROENTERITIS BETWEEN CITIES 1

ANNEXE IV. MODELLING THE REMOVAL AND REVERSIBLE IMMOBILIZATION OF MURINE NOROVIRUSES IN A PHAEOZEM UNDER VARIOUS CONTAMINATION AND RINSING CONDITIONS 1

ANNEXE V. REVERSIBLE IMMOBILIZATION AND IRREVERSIBLE REMOVAL OF VIRUSES IN SOILS OR MIXTURES OF SOIL MATERIALS; AN OPEN DATA SET ENRICHED WITH A SHORT REVIEW OF MAIN TRENDS 1

ANNEXE VI. RÉSUMÉ VULGARISÉ DE LA THÈSE 1

Liste des figures

Figure 1: Répartition de phénomènes climatiques extrêmes à l'échelle mondiale : a) Intensité des phénomènes de sécheresses illustrée en nombre de mois par an durant lesquels le volume d'eau prélevé dépasse le volume d'eau renouvelable (Mekonnen & Hoekstra, 2016) ; b) Évolution du temps entre épisodes de crues d'ici à 100 ans (Hirabayashi *et al.*, 2013)..... 2

Figure I.1: Pourcentage des eaux usées traitées et non traitées selon le niveau de revenu des pays pour l'année 2015 (Sato *et al.*, 2013; World Water Assessment Programme, 2017)..... 7

Figure I.2: Réutilisation globale des eaux usées traitées (part de marché par domaine d'utilisation), tiré de (Lautze *et al.*, 2014)..... 8

Figure I.3: Volumes d'eaux usées réutilisées en Europe en 2000 et prévisions à l'horizon 2025 (Raso, 2013)..... 9

Figure I.4 : Description des caractéristiques de quelques virus entériques et d'un virus modèle correspondant. 13

Figure I.5: Modélisation d'excrétion virale durant une infection à norovirus GII. Les courbes rouge et orange décrivent respectivement la médiane et les intervalles à 95% de l'excrétion. a) Cas d'un patient symptomatique. b) Cas d'un patient asymptomatique (Teunis *et al.*, 2015)..... 21

Figure I.6: Forces et interactions moléculaires entrant en jeu dans le cadre de la théorie DLVO étendue. Une énergie potentielle totale, issue de la somme des forces en présence (courbe orange), négative engendre une attraction contrairement à une énergie positive 29

Figure I.7: Abattements viraux dans les sols en fonction des teneurs en argiles telles que rapportés dans la littérature (Tesson & Renault, 2017)..... 30

Figure II.1: Map of the study area delineating the sewage collection basin, the area irrigated by wastewater and the sampling points. *(Punctual sampling of vegetables and borehole water were performed in some vegetable gardens near the Artière river, boreholes being located at about 50 to 300 m of the Artière bank)*..... 38

Figure II.2 : Comparison between actual daily data concerning all acute gastroenteritis (AGE) and the trends reflected by 7 d sliding averages..... 42

Figure II.3 a-c: Seasonal variations in acute gastroenteritis with medical prescriptions. (a) acute gastroenteritis for each age group; (b) probabilities of vomiting and/or having diarrhea for the all age groups; and (c) probabilities of vomiting for each age group..... 49

Figure II.4 a-c: Simulation from epidemiologic data of (a) the proportion of viral acute gastroenteritis, (b) the daily number of viral acute gastroenteritis, and (c) the concentration of viruses within raw wastewater at the inlet of the treatment plant. (Day 0: 1st September 2015). 50

Figure II.5 a-d: Measured virus concentrations in (a) raw wastewaters, (b) treated wastewater at the outlet of the treatment plant, (c) in the Artière River downstream treated wastewater discharge, and (d) in the Allier River upstream the confluence with the Artière River..... 53

Figure II.6 a-f: Concentrations in the Artière River downstream wastewater discharge (x-axis) vs concentrations in the Allier River upstream the confluence with the Artière River (y-axis) of (a) noroviruses GI, (b) noroviruses GII, (c) rotaviruses, (d) enteroviruses, (e) adenovirus, and (f) *Escherichia coli*. 54

Figure II.7 : Comparisons of simulated total daily virus shedding based on epidemiologic data to experimental equivalent virus concentrations assuming their following expression $veq, d = 10 - 2 \times NoGII, d + 5.8\ 10 - 4 \times RoV, d + 4.6\ 10 - 5 \times AdV, d$ (Day 0: 1st September 2015)..... 57

Figure II.8 : Comparisons of simulated virus concentrations in the Artière River downstream the discharge of wastewater, when neglecting the virus contamination of the Artière River upstream the wastewater discharge, to the sum of weighted measured virus concentrations. (Virus contamination of the Artière River upstream wastewater discharge could have increased simulated equivalent virus concentrations between about $9\ 10^{+1}$ and $1\ 10^{+3}$ copies.L^{-1}, except the 1st September 2015 where the increase in simulated value reached $7\ 10^{+4}$ copies.L^{-1}). (Day 0: 1st September 2015). 59

Figure II.9 : Simulated differences in equivalent virus concentrations in the Allier River between downstream and upstream the confluence with the Artière River for three scenarios of Allier viral contamination upstream the confluence: scenario 1: zero pollution; scenario 2: 10^{+4} copies.L^{-1}; scenario 3; equivalent virus concentration equal to 0.75 times the concentration in the Artière River downstream wastewater discharge. 60

Figure II.10 : Comparison of the simulated impacts of treated wastewater management for January and July 2015 months according to the three following scenarios: (i) treated wastewater discharged entirely in the Artière River, (ii) present withdrawal of treated wastewater operated for the *'ASA Limagne Noire'* association, and (iii) daily withdrawal of 50% of treated wastewater to fill reservoirs throughout the year. 62

Figure III.1 : Experimental design of the study. GW: groundwater, TWW: treated wastewater, FA: fulvic acids. 73

Figure III.2: Experimental setup used for the monitoring of virus immobilisation (flow according to the red path) and virus remobilisation (flow according to the green path.) 74

Figure III.3 a-b: Experimental data, and simulations $C_r(t = 0) = 1.82 \times 10^6$ copies.mL^{-1}, irreversible virus immobilization with $k_i = 1.23 \times 10^{-4}$ mL.g^{-1} s^{-1} and reversible immobilization satisfying Freundlich isotherm with $k_F = 1120$ and $n_F = 1.53$ for S_r in copies.g^{-1}. (a) one-week virus immobilization for artificial soil solution as circulating solution; (b) 7 hours remobilizations after 1, 2, 3 and 7-day immobilizations. 80

Figure III.4: Quantities of viruses recovered from the sampled soil solution (Qf samples), the reservoir (Qf reservoir) and the soil aggregate columns (Qf column), compared with initial virus quantity contaminating the reservoir (Q0). 81

Figure III.5: Estimated quantities of viruses released at the outlet of soil columns after the first half-hour of remobilization experiments with artificial soil solution, as a function of the final virus concentration during preliminary immobilization. Red dot shows the impact of removing one outlier value not taken into account to characterize the Freundlich isotherm... 82

Figure III.6: Experimental data for 7 hours virus remobilization experiments after a partial desaturation of the soil columns for 1 day (column DS1d) with final $\omega = 0.36$ g g^{-1} or 2 days (column DS2d-a) with final $\omega = 0.24$ g g^{-1} and column DS2d-b with final $\omega = 0.17$ g g^{-1}. (Simulations were performed using $C_r(t = 0)$, k_i, k_F and n_F estimated for immobilization and remobilization in columns always saturated (see values in caption of **Figure 3a, b.**)) 84

Figure III.7: Experimental data for 24 hours virus immobilization experiments for circulating artificial soil solution initially enriched with 10 mM of $MgCl_2$. (Simulations were performed without fitting to experimental data, assuming a partial decrease in [Mg^{2+}]) due to exchange with other cations retained on cation exchange sites of clays and organics 85

Figure III.8: Experimental virus remobilization experiments for circulating artificial soil solution, autoclaved wastewater, and groundwater, after a first virus immobilization for soil aggregate columns at 20°C and exposed to contaminated artificial soil solution. 86

Liste des photos

Photo III.1 a-b: (a) Experimental setup used for the monitoring of virus immobilisation, and (b) details of the column filled with soil aggregates. 74

Liste des tableaux

Tableau I-1 : Description des caractéristiques épidémiologiques des principaux virus entériques retrouvés en milieu hydrique (adapté de Le Guyader et al. (2014)) 18

Tableau I-2: Taux d'atteinte médian des infections (et décès) pour 100 000 habitants associées aux gastroentérites selon l'agent étiologique et la région du monde (tiré de Kirk et al. (2015)) 19

Tableau I-3: Abattements moyens sur les concentrations en Norovirus réalisés en station de traitement des eaux usées en fonction des technologies d'assainissement utilisées.23

Table II-1: Statistical data on acute gastroenteritis (AGE) leading to physician consultation and medical prescription as a function of the age group over the September 1st 2015 - August 31st 2016 period. 47

Table II-2 : Ratios of concentrations in the Artière River downstream wastewater discharge to Artière River near the confluence with Allier River. (- ratio not calculated when viruses were not detected either downstream wastewater discharge or near the confluence with the Allier River). 55

Table II-3 : Ratio of Virus or E. coli flows in the Allier River downstream the confluence with the Artière River to the sum of Virus or E. coli flows in the Allier River upstream the confluence and in the Artière River downstream wastewater discharge. (- ratio not calculated when viruses were not detected in one of these compartments). 56

Table III-1 : Main features of reversible immobilization models retained in published papers. 70

Table III-2 : Composition of circulating solutions 72

Liste des abréviations

ADN : Acide désoxyribonucléique

AdV : Adénovirus

ARN : Acide ribonucléique

DALY : Disability-Adjusted Life Years (Années de vie en bonne santé perdues)

ELISA : Enzyme-linked immunosorbent assay

EV : Entérovirus

GC : Copies génomiques

GEA : Gastro-entérite aigüe

MNV : Murine norovirus

NoV GI : Norovirus humain, génogroupe I

NoV GII : Norovirus humain, génogroupe II

NPP : Nombre le plus probable

OMS : Organisation mondiale de la santé

ONU : Organisation des Nations Unies

PCR : Polymerase chain reaction (réaction en chaîne par polymérase)

PIE : Point isoélectrique

QMRA : Quantitative Microbial Risk Assessment (Analyse quantitative des risques microbiologiques)

QVRA : Quantitative Viral Risk Assessment (Analyse quantitative de risques viraux)

REU : Réutilisation des eaux usées

RT-PCR : Reverse transcriptase PCR (transcription inverse puis réaction en chaîne par polymérase)

RV : Rotavirus

SRAS : Syndrôme respiratoire aigu sévère

VHA : Virus de l'Hépatite A

VIH : Virus de l'immunodéficience humaine

WWTP : Wastewater treatment plant

Introduction générale

Le monde est confronté à des problèmes croissants relatifs aux ressources en eaux douces exploitables, qui représentent moins de 1% de l'eau terrestre (Cai *et al.*, 2015; Comprehensive Assessment of Water Management in Agriculture *et al.*, 2007; Escobar & Schäfer, 2009; Kumar, 2015; Lazarova *et al.*, 2013; Scheierling *et al.*, 2010). Ces problèmes sont illustrés par des phénomènes de sécheresses récurrents et de baisse du niveau des nappes souterraines comme au Moyen-Orient (Qadir *et al.*, 2010), en Californie (Wilson *et al.*, 2016) et dans les pays méditerranéens (Fatta & Anayiotou, 2007; García-Ruiz *et al.*, 2011; Kellis *et al.*, 2013) mais aussi, depuis plusieurs années, au cœur du territoire français (Mourey & Vernoux, 2000). Ces problèmes s'accompagnent aussi d'une pollution (chimique, biologique et physique) croissante des différents compartiments de l'eau comme en Amérique du Sud (Villar *et al.*, 2007; Zarate *et al.*, 2014) ou en Afrique (Taiwo, 2011; Taiwo *et al.*, 2012; Zinkina & Korotayev, 2014).

L'altération en quantité et en qualité des ressources en eaux douces résulte de plusieurs facteurs de pression anthropique qui vont en s'accroissant. La croissance démographique globale, comme par en exemple en Afrique du Nord (Qadir *et al.*, 2010) ainsi qu'en Jordanie (Alfarra *et al.*, 2011), s'accompagne de l'accroissement des rejets d'eaux usées et de déchets (Cleland, 2013; Wada & Bierkens, 2014). L'urbanisation croissante des populations, présente à l'échelle globale mais en particulier en Asie et en Afrique (DESA *et al.*, 2014; Kennedy *et al.*, 2012), provoque une concentration de la demande et des rejets d'eaux usées. Le réchauffement global provoque quant à lui une augmentation de la fréquence des événements climatiques extrêmes qui sont en mesure à la fois de diminuer le volume des réserves d'eau douce, à travers les sécheresses (**Figure 1a**), mais aussi d'en réduire la qualité, par la pollution apportée lors d'inondations (**Figure 1b**) et des entrées d'eau marines dans les aquifères d'eau douce (Cai *et al.*, 2015; Gleick, 2014; Hirabayashi *et al.*, 2013; Kløve *et al.*, 2014; Taylor *et al.*, 2012, 2012; Weinthal *et al.*, 2015). Enfin la diversification des usages de l'eau, avec notamment de plus en plus d'usages récréatifs (irrigations de golfs ou de parc, piscines privées …) participent à l'accroissement de la demande en eau (Tanaka *et al.*, 1998).

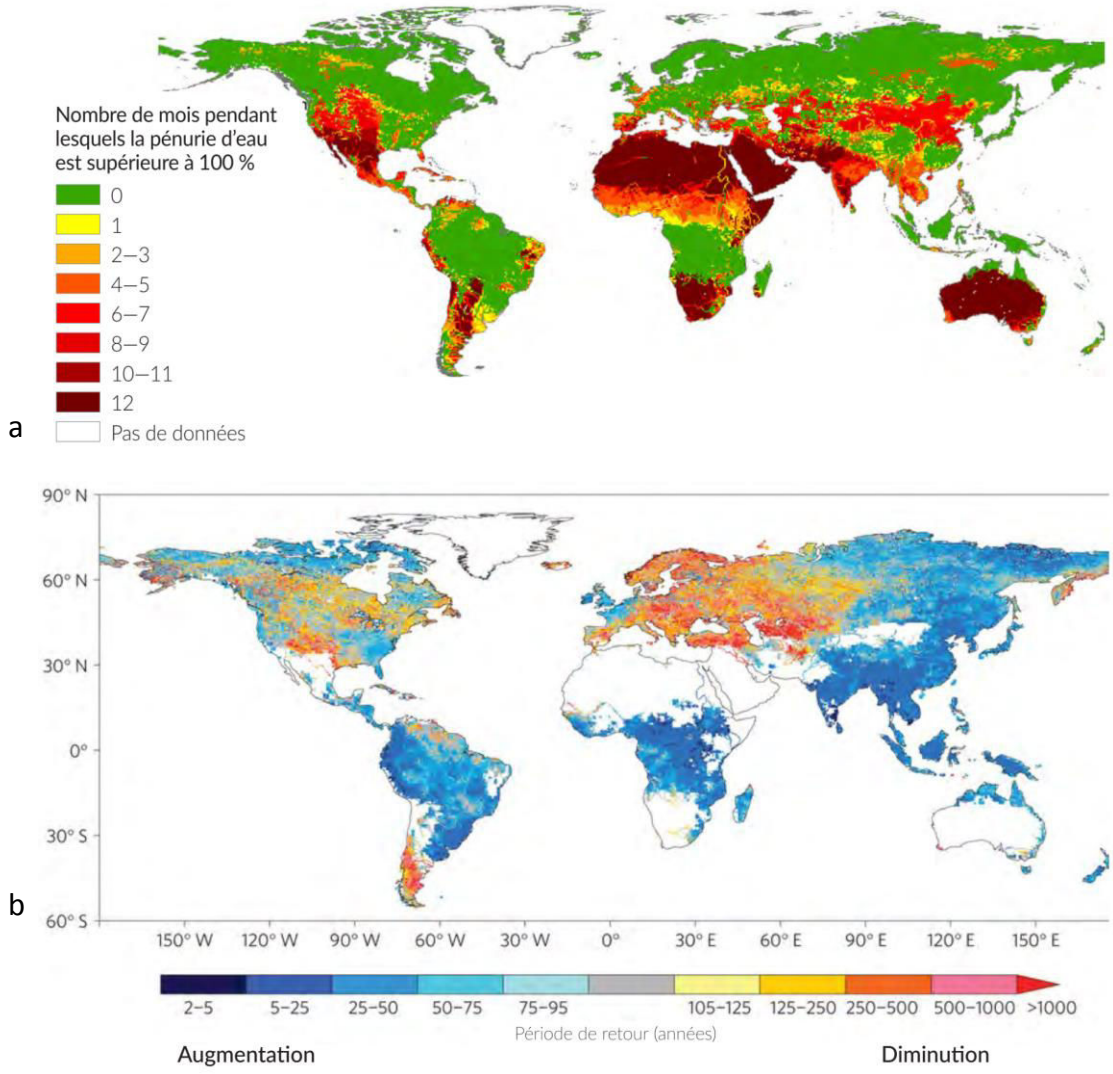


Figure 1: Répartition de phénomènes climatiques extrêmes à l'échelle mondiale : a) Intensité des phénomènes de sécheresses illustrée en nombre de mois par an durant lesquels le volume d'eau prélevé dépasse le volume d'eau renouvelable (Mekonnen & Hoekstra, 2016) ; b) Évolution du temps entre épisodes de crues d'ici à 100 ans (Hirabayashi *et al.*, 2013).

Afin de faire face à ces problèmes croissants de quantité et de qualité des ressources en eaux conventionnelles, plusieurs initiatives ont été prises, incluant la mise en place de politiques et de stratégies pour la réduction de la consommation d'eau (Craun *et al.*, 2010; Jiménez Cisneros & Asano, 2008) et pour la réduction de leurs contaminations (Craun *et al.*, 2010; Prüss-Ustün *et al.*, 2014). Plusieurs stratégies sont envisageables localement pour l'accroissement en quantité des ressources en eau : le transport d'eau sur de longues distances et l'exploitation de ressources en eau dîtes « marginales », i.e. la désalinisation d'eaux saumâtres et salées ainsi que le recyclage des eaux usées d'origine industrielle, urbaine ou domestique (World Water Assessment Programme, 2017). Ces ressources nécessitent un traitement préliminaire. Les eaux usées sont ainsi déjà exploitées pour de l'irrigation (municipale ou agricole) (Jaramillo & Restrepo, 2017), de la recharge d'aquifères, des activités industrielles (Van der Bruggen, 2010) telles que le refroidissement d'installations, et

parfois la potabilisation comme c'est déjà le cas en Israël (Cai *et al.*, 2015; Leverenz *et al.*, 2011; Navarro *et al.*, 2015; OCDE, 2012a; Weinthal *et al.*, 2015), en Namibie sur le site de Windhoek (du Pisani, 2006; Lahnsteiner & Lempert, 2007) et en Californie (Shipps, 2013; Steirer & Thorsen, 2013). Le traitement subi par les eaux avant recyclage est extrêmement variable ; il dépend pour partie de la richesse du pays (Sato *et al.*, 2013; World Water Assessment Programme, 2017). En pratique, cela peut aller d'un traitement nul jusqu'à un traitement permettant la potabilisation des eaux usées. La répartition des volumes d'eau usée en fonction de ces secteurs diffère grandement en fonction des pays mais globalement le secteur le plus consommateur d'eaux usées est celui de l'irrigation agricole (Bixio *et al.*, 2008; Van der Bruggen, 2010; World Water Assessment Programme, 2017).

Cette pratique ancienne est aujourd'hui présente à travers le monde (Angelakis *et al.*, 2005; Angelakis & Snyder, 2015) avec plus ou moins d'importance. Si quelques villes et pays, comme Israël (OCDE, 2012a; Weinthal *et al.*, 2015), l'Afrique sub-saharienne (Qadir *et al.*, 2010), la Chine (Yi *et al.*, 2011) et les domaines irrigués en périphérie de la ville de Mexico (Jiménez, 2005; Jiménez-Cisneros & Chávez-Mejía, 1997) apparaissent comme de grands consommateurs d'eau usée réutilisée, cette pratique peine à se développer dans les pays qui ne sont pas soumis à un stress hydrique modéré, comme la France par exemple. En effet, même si certaines régions françaises sont soumises occasionnellement à des épisodes plus ou moins intenses et longs de canicules-sècheresses (García-Ruiz *et al.*, 2011) qui s'accompagnent de restrictions d'usages de l'eau, l'indice de stress hydrique de la France (inférieur à 20%, i.e. le rapport entre consommation annuelle d'eau et ressources en eaux facilement reconstituables) est considéré comme un indice faible (OCDE, 2012b) ce qui, couplé à une législation particulièrement délicate en matière de dossier préalable à l'obtention d'une autorisation préfectorale et de distances à respecter pour l'irrigation par aspersion afin d'éviter les contaminations par voie aérienne (Ministère de la Santé et des Sports, 2010, 2014, 2016; Molle *et al.*, 2016) et un manque de connaissance sur les risques sanitaires associés à la pratique (Toze, 2006), freine la mise en place de projets de réutilisation des eaux usées (Bixio *et al.*, 2006).

La réutilisation en irrigation agricole présente potentiellement des risques qu'il convient d'évaluer et de comparer à ceux résultant de scénarios de rejets des eaux usées traitées dans l'environnement comme le rejet de l'intégralité des eaux usées dans l'environnement ou la réutilisation d'une partie d'entre elles dans diverses filières (irrigation agricole ou municipale, refroidissement d'installations industrielles ou potabilisation par exemple). Ils incluent des risques environnementaux, des risques agricoles et des risques sanitaires (Qu *et al.*, 2016; Toze, 2006), associés au contenus de ces eaux, même traitées, en contaminants chimiques (Chefetz *et al.*, 2008) et biologiques, notamment en virus entériques pathogènes de l'Homme (Lim *et al.*, 2016; Pouillot *et al.*, 2015; Zhou *et al.*, 2015).

Les virus entériques pathogènes de l'Homme sont les agents étiologiques majeurs des maladies entériques d'origine hydrique (Guzman-Herrador *et al.*, 2015; Moreira & Bondelind, 2017; Radin, 2014) et alimentaire à travers le monde (Kirk *et al.*, 2015; Pires *et al.*, 2015),

parmi lesquelles on retrouve les gastroentérites, les hépatites ou la poliomyélite. Les espèces virales à l'origine de ces maladies sont nombreuses mais certaines ressortent particulièrement, parmi lesquelles se retrouvent les norovirus, les rotavirus, le virus de l'hépatite A, les entérovirus (qui comprennent les coxsackievirus et les poliovirus) et les adénovirus. Ces pathogènes sont actuellement reconnus comme étant des contaminants à rechercher dans les eaux de boisson par l'American Water Works Association et l'Agence américaine de protection de l'environnement depuis 2009 (Hoffman *et al.*, 2009; US EPA, 2014) et dans les produits alimentaires par le *Codex Alimentarius* (FAO, 2012).

S'il est aujourd'hui reconnu que les virus entériques de l'Homme sont particulièrement résistants aux traitements réalisés en station d'épuration (Campos *et al.*, 2016; La Rosa *et al.*, 2010; Pouillot *et al.*, 2015; Sano *et al.*, 2016), certaines zones d'ombres demeurent quant à leur devenir une fois libérés dans l'environnement. Bien que beaucoup d'études ont porté sur la survie et le transport des virus dans des contextes très artificialisés (notamment pas les matériaux "sols", leur saturation en eau en général …), peu d'études ont traité de la survie et du transport des virus après qu'ils aient été déposés au sol lors d'une irrigation par des eaux usées dans des conditions proches de la réalité (Tesson & Renault, 2017). Par ailleurs, même si la contamination des rivières lors de rejets d'eaux usées (Prevost *et al.*, 2015b; Wyn-Jones *et al.*, 2011) et leur survie lors de rejets d'eaux usées a été largement étudiée (Gerba, 2007; Ogorzaly *et al.*, 2010; Prevost *et al.*, 2015b), peu d'études sont allées jusqu'à modéliser la contamination à la source, c'est-à-dire dès l'excrétion par les individus malades afin de prévoir les concentrations en rivières et les risques associés (Petterson *et al.*, 2016).

Il reste nécessaire de pouvoir mettre en place un modèle permettant de simuler, à partir de données épidémiologiques, le devenir environnemental des virus depuis leur excrétion jusque leur arrivée dans les cours d'eaux, lors de rejets, ou dans le continuum « sol-plante » et le risque de passage à la nappe souterraine, lors d'une réutilisation.

Les objectifs de cette thèse étaient les suivants :

- proposer une méthode permettant de passer de données épidémiologiques sur les gastroentérites aiguës à des nombres journaliers de nouveaux cas de gastroentérites d'étiologie virale et aux quantités de virus arrivant avec les eaux usées brutes en station d'épuration ;
- caractériser et décrire le devenir environnemental des virus dans le continuum « station d'épuration – eaux douces » lorsque les eaux usées sont rejetées après traitement ;
- caractériser et décrire le devenir d'un virus modèle dans le sol lorsque les eaux usées sont réutilisées en irrigation agricole

Chapitre I

Synthèse bibliographique

1. Les eaux usées et leur réutilisation

Les eaux usées sont définies par l'ONU comme « *une combinaison d'un ou de plusieurs des éléments suivants : les effluents domestiques constitués d'eaux-vannes (excréments, urine, boues fécales) et d'eaux grises (eaux usagées provenant du lavage, de la lessive et du bain) ; les eaux provenant des commerces et institutions, y compris les hôpitaux ; les effluents industriels, les eaux pluviales et autres eaux de ruissellement urbain ; les eaux de ruissellement agricole, horticole et aquacole* » (Programme mondial pour l'évaluation des ressources en eau 2017 ; Raschid-Sally et Jayakody 2009).

Lors de leur rejet les eaux usées brutes peuvent avoir plusieurs destinations. À l'échelle mondiale, on estime à 80 % la proportion des eaux usées rejetées dans l'environnement sans traitement ou après un traitement physique (World Water Assessment Programme, 2017), cette valeur évoluant en fonction des revenus des pays (**Figure I.1**). Ces rejets affectent l'environnement, et en particulier les ressources en eaux douces, à travers les pollutions chimique, physique et biologique qu'elles apportent. Ces pollutions entraînent la diminution simultanée du volume des ressources en eau facilement exploitables et leur qualité. Les conséquences de ces phénomènes s'observent régulièrement à travers le monde, notamment dans les pays en voie d'industrialisation lors de catastrophes naturelles. Un exemple récent est celui de l'épidémie de choléra survenue en Haïti en 2010 à la suite d'un tremblement de terre et à l'explosion des pressions exercées sur les faibles ressources en eau potable disponibles (Piarroux *et al.*, 2011). Dans d'autres régions du monde, telles que le Moyen-Orient (Gleick, 2014; Weinthal *et al.*, 2015) ou l'Afrique sub-saharienne (Couttenier & Soubeyran, 2014), la raréfaction des ressources en eau conventionnelles favorise la naissance de conflits armés (Salehyan 2014 ; Brown et Matlock 2011). Au regard des rejets dans l'environnement, les eaux usées peuvent apporter des pollutions chimique et physique qui engendrent, entre autres exemples, une perte de biodiversité dans les rivières et des phénomènes d'eutrophisation (Thomas *et al.*, 2010). Néanmoins il existe des solutions permettant de réduire à la fois l'impact des rejets et l'importance des prélèvements, parmi lesquelles la réutilisation des eaux usées pour diverses activités (refroidissement industriel, recharge d'aquifères, activités de loisirs, irrigation agricole, …)

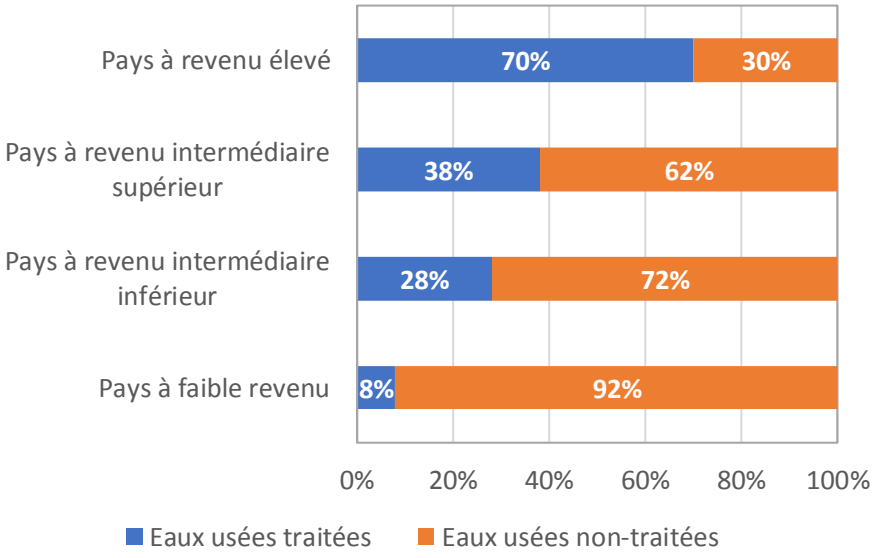


Figure I.1: Pourcentage des eaux usées traitées et non traitées selon le niveau de revenu des pays pour l'année 2015 (Sato *et al.*, 2013; World Water Assessment Programme, 2017).

La réutilisation des eaux usées est une pratique reconnue et largement répandue à travers le monde. Les premières traces remontent à l'âge de Bronze et peuvent aujourd'hui être retrouvées autant en Europe de l'ouest qu'en Afrique, en Asie ou en Amérique (Agrafioti & Diamadopoulos, 2012; Angelakis & Durham, 2008; Angelakis & Snyder, 2015; Asano & Levine, 1996; Jaramillo & Restrepo, 2017; Tsagarakis *et al.*, 2004; Yi *et al.*, 2011). De nos jours, l'irrigation agricole représente le secteur le plus consommateur à la fois d'eaux douces avec 70% des prélèvements ce qui correspond à 2769 $km^3.an^{-1}$ (Earthscan 2011 ; AQUASTAT 2016) et d'eaux usées avec 32% des parts de marché (**Figure I.2**). Ce type d'irrigation réutilise des eaux usées qui sont en grande majorité des eaux usées brutes (environ 10 fois plus que les eaux usées traitées selon un rapport de l'ONU (World Water Assessment Programme, 2017)) et ce principalement en Asie du Sud-Est, en Afrique et en Amérique du Sud (Jaramillo et Restrepo 2017 ; Jiménez Cisneros et Asano 2008). Dans ce cas, l'eau usée n'est pas traitée chimiquement ou biologiquement et est utilisée par aspersion ou en irrigation gravitaire des cultures. Cette pratique donne d'excellents résultats mais présente des risques sanitaires et environnementaux importants (Keraita, 2008). En parallèle, l'irrigation agricole réutilise des eaux usées ayant subi une série de traitements biologiques et/ou physicochimiques, en station d'épuration, afin de réduire les concentrations en polluants divers. Cette deuxième option correspond à la pratique privilégiée en Europe et autour de la Méditerranée, dans certains états des U.S.A. et en Australie (Bixio *et al.*, 2006; Jiménez Cisneros & Asano, 2008; Kellis *et al.*, 2013; Lim *et al.*, 2016) ainsi qu'en Israël qui réalise jusque 50% de ses irrigations agricoles avec des eaux usées réutilisées (OCDE, 2012a).

La réutilisation dans le cadre de l'irrigation agricole présente de nombreux intérêts. Le premier d'entre eux est environnemental car l'irrigation par les eaux usées va apporter à l'exploitant une ressource en eau supplémentaire stable tout au long de l'année en périphérie

de centres urbains, qui rejettent des volumes réguliers d'eaux usées. Ceci permet à l'agriculteur de réduire ses prélèvements dans les réserves d'eaux douces et d'éviter le retour direct des eaux usées à l'environnement.

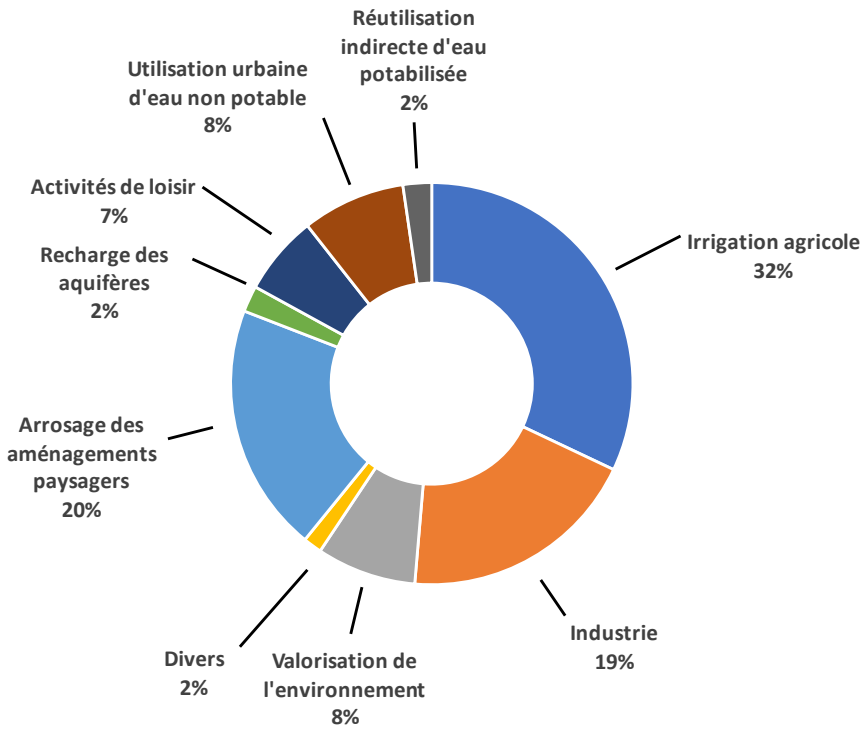


Figure I.2: Réutilisation globale des eaux usées traitées (part de marché par domaine d'utilisation), tiré de (Lautze *et al.*, 2014).

Dans les pays où la réutilisation est surtout basée sur les eaux usées brutes, cette ressource en eau dite marginale est riche en micronutriments (en particulier N et P) ce qui donne accès à une certaine fertilisation ou permet un usage réduit des fertilisants commerciaux. Leur réutilisation pour l'irrigation agricole à proximité de Mexico a permis par exemple une augmentation significative des rendements entre 67 % (blé) et 150 % (maïs) (Jiménez, 2005). Dans les zones tropicales, les eaux usées permettent par ailleurs de réaliser plusieurs cultures sur une même parcelle durant une année (Keraita, 2008). Ces avantages permettent d'apporter une sécurité alimentaire aux populations en périphérie et dans les centres urbains, en particulier dans les pays en voie d'industrialisation.

Par ailleurs, la réutilisation des eaux usées, en majorité d'origine domestique, amène à réduire les apports de de contaminants chimiques et biologiques aux ressources en eau douce en redirigeant ces contaminants vers les parcelles agricoles ; une partie pourra être retenue et/ou détruite une fois apportée au sol ou au niveau de la plante

Néanmoins la réutilisation des eaux usées en agriculture ne s'impose d'elle-même que dans les cas où la demande en eau est très forte et sa disponibilité très faible, dans les zones régulièrement soumises à des épisodes de sécheresse comme en Afrique sub-saharienne par

exemple (Qadir *et al.*, 2010) ou en région méditerranéenne (Kellis, Kalavrouziotis, et Gikas 2013 ; Fatta et Anayiotou 2007 ; Pedrero et al. 2010 ; Tsagarakis, Dialynas, et Angelakis 2004 ; Bixio et al. 2006). En dehors de ces cas où l'exploitation de ressources marginales devient nécessaire, les projets de réutilisation des eaux usées peinent à se développer du fait du manque d'urgence (Bixio *et al.*, 2008) ou de son non-ressenti (**Figure I.3**), comme en Bulgarie (Hochstrat *et al.*, 2005, 2006) dont l'indice de stress hydrique dépasse 60% (Raso, 2013).

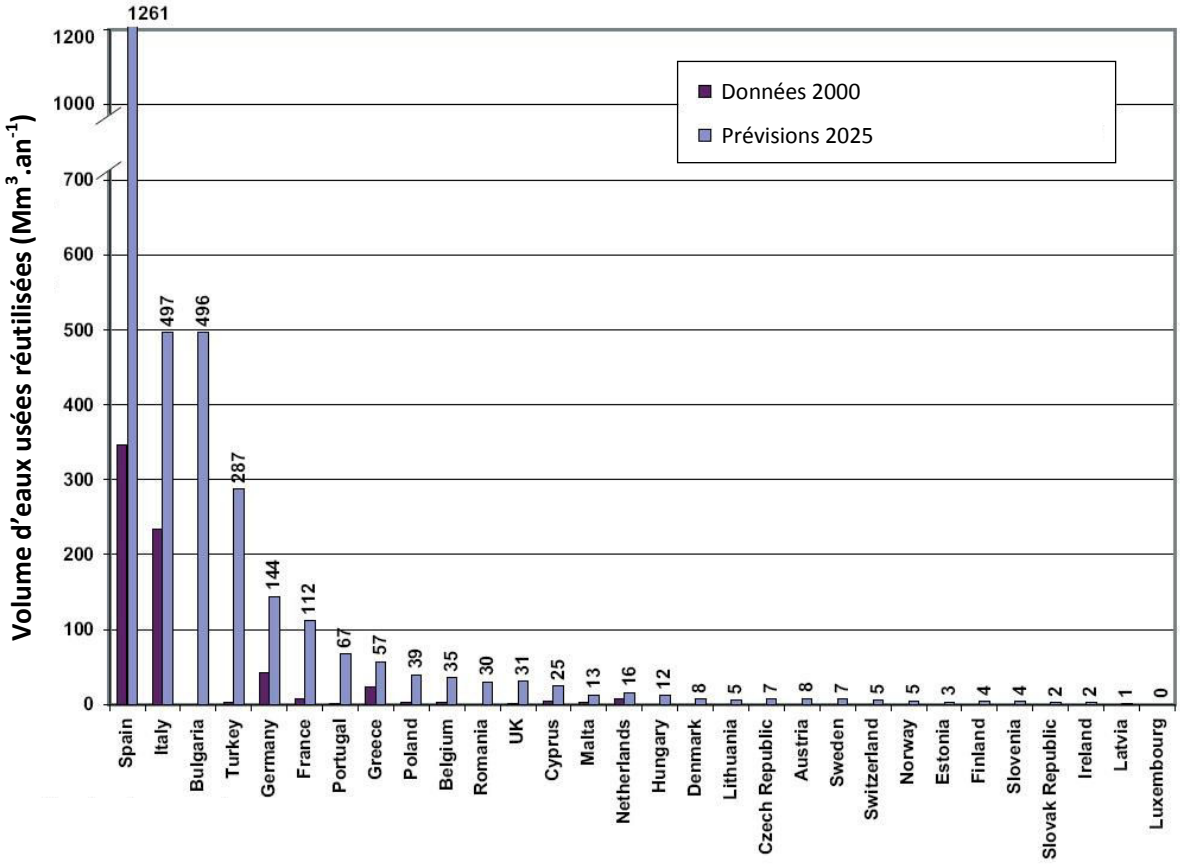


Figure I.3: Volumes d'eaux usées réutilisées en Europe en 2000 et prévisions à l'horizon 2025 (Raso, 2013).

Un autre facteur pouvant freiner la réutilisation des eaux usées en agriculture concerne l'acceptabilité sociale de la pratique, autant chez les exploitants que chez les consommateurs, qui peuvent avoir des appréhensions quant à consommer les produits concernés par ce type d'irrigation (Drechsel *et al.*, 2015). En effet, la réutilisation des eaux usées nécessite dans certains pays de se conformer à des réglementations plus ou moins contraignantes qui, par conséquent, peuvent engendrer des coûts de mise en place prohibitifs (California Department of Substances Control, 2014, p. 22). À ceci peut aussi s'ajouter des préjugés résultant des problèmes associés aux anciennes politiques d'épandage de boues d'épuration, qui ont été sources de pollution des sols aux métaux lourds, et aux odeurs associées à certains sites (Angelakis & Snyder, 2015).

Le dernier frein au déploiement des politiques de réutilisation des eaux usées concerne le manque de connaissances relatif au devenir d'une partie des contaminants une fois ceux-ci déversés dans l'environnement, en particulier concernant les virus entériques et les substances organiques émergentes. Ces connaissances permettant de proposer un cadre légal (réglementations, normes) basé sur une évaluation des risques pour les populations, portant à la fois sur la qualité des eaux employées, les durées et les périodes d'irrigation. Il existe ainsi trois types principaux de réglementations. La première repose sur une approche pragmatique (et empirique) des risques, à partir notamment de données épidémiologiques. Cette approche préconisée par l'O.M.S. jusqu'en 2006 (World Health Organization, 2006) est celle adoptée par la France et l'Espagne, dont la région autonome de Valencia par exemples (Bixio et al. 2006 ; Raso 2013 ; Kellis, Kalavrouziotis, et Gikas 2013). Une deuxième approche préconise le « risque zéro » ; les règlementations et normes s'appuient alors sur un contrôle strict de la qualité des eaux usées, parfois fixée à un niveau au-dessus de celui des eaux d'irrigation conventionnelles. L'option prise par la Californie (avec le Title 22 (California Department of Substances Control, 2014) et le Purple Book (US FDA, 2017)) est typique de ce type d'approche ; mais cette option a aussi été retenue par d'autres pays ; parmi lesquels l'Italie (Lopez *et al.*, 2006; Verlicchi *et al.*, 2012) , Chypre (Kathijotes & Panayiotou, 2013; Raso, 2013) et l'Australie (Horne, 2016) ; même si certains d'entre eux se pose la question de son intérêt (coût éventuellement élevé des traitements additionnels au regard du gain monétaire associé à la baisse du nombre d'individus malades). Les limites des deux options précédentes (empirisme et/ou coût) ont poussé l'O.M.S. à proposer dès 2006 la mise en place de règlementations et normes basée sur l'évaluation quantitative des risques microbiologiques (QMRA) et leur quantification en nombre d'années de vie perdues (DALY) (World Health Organization, 2006). Le dernier type de réglementation est considéré comme « pragmatique » et est actuellement en vigueur en Australie (Biotext Pty Ltd *et al.*, 2006). Cette réglementation se base sur une gestion de la réutilisation avec pour objectif une augmentation raisonnable du risque pour la santé publique et l'environnement plutôt que d'essayer d'atteindre un risque nul. Pour cela il est nécessaire de bien identifier et quantifier les risques ainsi que les populations et zones dites « sensibles », c'est-à-dire dont la santé est plus fragile que la moyenne (femmes enceintes, enfants, personnes âgées, immunodéprimées ou transplantées (Reynolds *et al.*, 2008)) et de réaliser une analyse quantitative du risque microbien (QMRA) associée à la mise en œuvre de la pratique (Mara *et al.*, 2007).

En effet des risques sanitaires associés aux eaux usées, même traitées en station d'épuration, persistent. Une partie des polluants chimiques et biologiques initialement présents dans les eaux usées brutes n'est pas détruite ou retenue par les traitements réalisés en station. Ainsi même si les métaux lourds (Afifi *et al.*, 2011) sont très largement retenus lors des traitements, les eaux rejetées sont encore chargées en substances organiques émergentes (produits pharmaceutiques, composés associés aux produits de soins personnels, phtalates, des pesticides urbains et divers autres composés (HAP, bisphénol A …)), dont certains peuvent être considérés comme des perturbateurs endocriniens (Jarque et al. 2015 ; Di Poi et al. 2014) ou participer à l'émergence de souches bactériennes antibiorésistantes (Rodriguez-Mozaz *et*

al., 2015). Si les protozoaires et œufs d'helminthes sont particulièrement bien éliminés lors du processus d'épuration des eaux (Nasser *et al.*, 2016), les bactéries et les virus entériques de l'Homme sont quant à eux présents en concentrations suffisamment élevées en amont du traitement pour toujours représenter un risque sanitaire important en sortie de station (Campos *et al.*, 2016; Zhou *et al.*, 2015). Ainsi, malgré des taux d'abattements moyens en station de l'ordre de 0,1 à 3 $\log_{10}$ (Campos et al. 2016 ; Pouillot et al. 2015 ; Nasser et al. 2016 ; La Rosa et al. 2010 ; Sano et al. 2016 ; Zhou et al. 2015), les eaux usées traitées présentent encore, en sortie des stations, des contaminants biologiques. Des concentrations de l'ordre de $10^{1,8}$ NPP.100 mL^{-1} pour les *E. coli* et de $10^{6.3}$ $gc.mL^{-1}$ pour les Norovirus ont été calculées en sortie de stations de traitement autour de la ville de Rome, après avoir subi des abattements respectifs de 98,5% et 78,4% (La Rosa *et al.*, 2010).

La persistance de ces bactéries et virus et leur dissémination lors d'irrigations introduit un risque de contamination aérienne lors d'aspersions (Girardin *et al.*, 2016), un risque de contamination des ressources en eau (nappes, rivières, captages d'eau potable…) (Jagai *et al.*, 2012; Jung *et al.*, 2011; Moreira & Bondelind, 2017; Murphy *et al.*, 2017; Prevost *et al.*, 2015b) et un risque de contamination des cultures, soit direct ou soit *via* le sol avec la possibilité d'internalisation de virus notamment *via* les racines (DiCaprio *et al.*, 2015; Yang *et al.*, 2017). Il en découle un risque sanitaire, avec des impacts plus extrêmes pour les populations sensibles telles que les personnes âgées, les femmes enceintes ou les personnes immunodéprimées (Reynolds *et al.*, 2008).

2. Les virus entériques pathogènes de l'Homme

a. Description générale des virus

Les virus sont des organismes parasites obligatoires : leur réplication ne peut avoir lieu qu'au sein de leur hôte contaminé. Par conséquent, les virus entériques pathogènes de l'Homme détectés dans l'environnement y ont forcément été rejetés avec les selles d'un hôte contaminé. Bien qu'extrêmement différents les uns des autres, la majeure partie des virus entériques pathogènes de l'Homme partagent des caractères précis :

- ils possèdent un matériel génétique, porté une molécule d'acide nucléique (ADN ou ARN) mono- ou bicaténaire ;
- ce matériel génétique est protégé par une capside, structure protéique chargée à la fois de protéger le génome des attaques extérieures et d'assurer tout ou partie du pouvoir infectieux du virus. L'ensemble est nommé « nucléocapside » ;
- pour infecter un hôte, les protéines de surface de la particule virale doivent être reconnues par ces récepteurs de surface de la cellule hôte, par exemple les récepteurs HBGA, qui définissent le groupe histologique. Le tropisme du virus

correspond au type de cellule, et par conséquent à l'organe, que le virus est capable d'infecter. La spécificité de la liaison peut varier selon le virus.

- Un virus n'est capable de se répliquer qu'après avoir pénétré la cellule infectée et détourné son métabolisme cellulaire.

La plupart des virus entériques pathogènes de l'Homme sont des virus nus, i.e. constitués uniquement d'une nucléocapside. Celle-ci sera donc chargée à la fois de protéger le génome et d'assurer le pouvoir infectieux du virus. Quelques rares virus pouvant être considérés comme potentiellement entériques pathogènes de l'Homme sont des virus enveloppés, constitués d'une nucléocapside recouverte elle-même d'une bicouche lipidique, provenant le plus souvent de la membrane cellulaire de la dernière cellule infectée : c'est par exemple le cas de certains coronavirus qui peuvent être détectés dans les selles de certains malades (Jevšnik *et al.*, 2013).

En tant que parasites obligatoires, les virus ont évolué avec leur hôte, renforçant la spécificité de leur relation. Cette évolution conjointe est extrêmement ancienne et les a amenés à parasiter des représentants de tous les règnes du vivant, eucaryotes comme procaryotes ou acaryotes : les Animaux, les Plantes, les Champignons, les protistes, les Archées ; les Bactéries (avec les bactériophages) de même que les virus eux-mêmes par le biais des virophages (Scola *et al.*, 2008). Toutefois, la plupart des espèces virales ne peuvent infecter qu'un seul hôte, ou un très faible nombre d'espèces, mais il existe des exceptions telles que le virus du SRAS ou celui de la rage, responsables de zoonoses. Le virus de la mosaïque du tabac serait un autre exemple capable d'infecter plusieurs espèces végétales de la famille des *Solanaceae*.

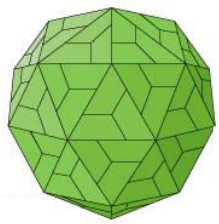
D'un point de vue général, les virus présentent une variété importante en termes de taille, de structure de la capside et du génome. Les particules virales, nommées virions, peuvent par exemple mesurer entre une dizaine de nanomètres pour les *Circovirus* et 1,5 µm dans le cas des *Pandoravirus* ; des virus d'amibes récemment découverts (Legendre *et al.*, 2014). Toujours en ce qui concerne la nucléocapside, celle-ci présente une grande diversité de structures pour lesquelles la classification s'est portée sur la symétrie de l'enveloppe protéique, composée de sous-unités nommées capsomères, permettant ainsi de définir trois grandes catégories :

- Les capsides à symétrie hélicoïdale dont le premier représentant est le virus de la mosaïque du tabac. La capside des virions est ici constituée d'un assemblage de capsomères prenant la forme d'un cylindre dont le volume est directement proportionnel au nombre de sous-unités constituant la structure. Certains virus à ARN pathogènes des Animaux sont connus pour dissimuler sous leur enveloppe une nucléocapside hélicoïdale. Par exemple les virus du genre *Betacoronavirus*, connus pour être responsables d'infections principalement respiratoires chez l'Homme, possèdent une nucléocapside en forme de tube de symétrie hélicoïdale.
- Les capsides à symétrie icosaédrale résultent d'un assemblage de sous-unités protéiques non-nécessairement identiques. Ces virus, le plus souvent de forme

sphérique, peuvent révéler une structure d'une très grande complexité en fonction de leur taille. Par exemple la capside des *Picornavirus* tels que les poliovirus mesurent environ 30 nm et composée d'un assemblage de 60 unités protéiques correspond aux structures icosaédrales les plus simples possibles tandis que la capside des *Adenovirus* est considérée comme un modèle de complexité avec un assemblage de 252 capsomères de deux types associés à 7 protéines de surface, pour une obtenir une particule virale nue d'une taille située entre 60 et 90 nm (**Figure I.4**). Certains de ces virus, comme les bactériophages T4 (**Figure I.4**) peuvent comporter une extension protéique, dans ce cas précis placée sous la tête du bactériophage et permettant l'injection du génome viral dans la bactérie cible.

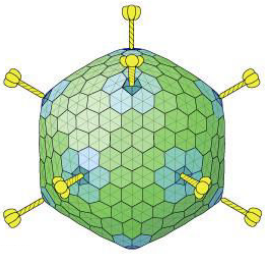
- Les capsides dîtes asymétriques sont en minorité chez les virus. Elles correspondent à des virus dont la structure est souvent encore mal comprise. Un exemple concerne les *Poxvirus*, qui se présentent sous la forme de virions de forme cylindrique d'environ 360 nm de long irrégulièrement recouvert de protrusions d'environ 3 à 5 nm.

Les virus entériques sont en très grande majorité des virus nus, à symétrie icosahédrale dont la taille peut varier entre 38 et 80 nm (**Figure I.4**). Leur génome peut être porté par un ou plusieurs molécules d'ADN ou ARN, de polarité positive ou négative.



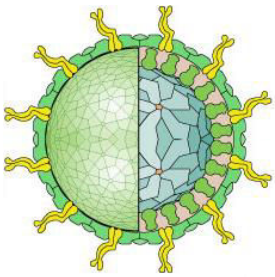
Norovirus

- Genre: *Caliciviridae*
- Virus nu
- Capside à symétrie icosahédrale
- Taille : 38-40 nm
- PIE = 5,5 – 6,9
- Génome ARN+ simple brin



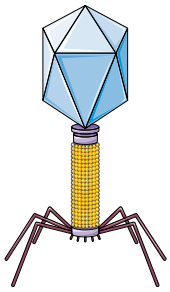
Adénovirus

- Genre: *Adenovirus*
- Virus nu
- Capside à symétrie icosahédrale
- Taille : 90 nm
- PIE = 4,5
- Génome ADN double brin



Rotavirus

- Famille: *Reoviridae*
- Virus nu
- Capside à symétrie icosahédrale
- Taille : 80 nm
- PIE = 8
- Génome ARN+ double brin



Bactériophage T4

- Genre: *T4-like viruses*
- Virus nu
- Tête à symétrie hélicoïdale
- Taille : 120 x 86 nm
- PIE = 2,0 ; 4,0 – 5,0
- Génome ADN double brin

Figure I.4 : Description des caractéristiques de quelques virus entériques et d'un virus modèle correspondant.

Enfin cette diversité se retrouve au sein du génome des virus. Celui peut être porté par une ou plusieurs molécules au choix d'ADN, comme chez les Adenovirus, ou d'ARN, comme observé chez les Influenzavirus et mesurer de 4 kB à plus de 2500 kB. Ce génome peut, dans le cas des virus, prendre une forme linéaire, circulaire voire fragmentée. Dans le cas des génomes ARN ceux-ci peuvent être lus dans le sens positif (5'- 3') ou négatif (3' - 5') avec dans ce dernier cas la nécessité de devoir être transcrit en une molécule d'ARN de polarité positive pour acquérir leur pouvoir infectieux. Le génome viral présente par ailleurs d'autres spécificités, en particulier en termes de plasticité. En effet, les virus ne possèdent pas de mécanismes de contrôle des erreurs de réplication ce qui peut amener à l'apparition de mutations dont une partie peut totalement modifier le comportement du pathogène, avec pour conséquences possibles une modification du tropisme du virus et par la même occasion des symptômes de la maladie, la possibilité de contourner l'immunité acquise de l'hôte voire d'être capable d'infecter de nouvelles espèces, comme cela a été le cas pour le virus du SRAS (Field, 2009)

Les virus ne sont pas visibles au microscope optique et ne peuvent pas être cultivés sur un milieu gélosé comme les Bactéries. La détection par sérologie fut la première technique développée durant la première moitié du XXe siècle. Elle est basée sur la réaction antigène viral/anticorps développée chez les patients immunocompétents lors de leur rencontre avec le pathogène. Cette technique demeure toujours employée de nos jours, notamment dans le cadre de la détection du VIH, des hépatites ou du virus de l'herpès ainsi que dans le cadre des tests ELISA ou d'immunofluorescence. Le premier isolement de virus sur culture cellulaire est réalisé en 1948 sur une souche de Poliovirus (Enders *et al.*, 1949). La méthode repose sur la mise en contact des particules virales avec une culture de cellules permettant la réplication du pathogène, le tout dans un milieu liquide sur un support physique. Cette méthode permet la production de virus *in-vitro* par des infections successives de tapis cellulaires sains puis lyse de ces dernières, recueil du surnageant de culture (contenant les particules nouvellement formées) et infection d'un nouveau tapis. Les cellules utilisées sont en majorité issues de lignées continues ou transformées c'est-à-dire issues de cellules tumorales ayant conservé les récepteurs de surface permettant l'infection et possédant une durée de vie illimitée. Il est aussi possible d'employer des cultures primaires (issues d'un organe prélevé, souvent au stade embryonnaire) ou issues de lignées semi-continues (stables génétiquement mais sénescentes après un certain nombre de cycles de réplication).

La fin du XXe siècle voit apparaitre la technique de détection par biologie moléculaire dite de PCR. Celle-ci permet la détection du génome ADN du virus *via* l'action d'une enzyme bactérienne, la polymérase. Celle-ci va réagir à une succession de cycles de températures qui permettront son accroche sur une région précise du génome ; guidée par des brins d'ADN conçus à cette fin ; puis le recrutement des nucléotides et leur polymérisation afin de créer un brin d'acide nucléique complémentaire du premier. La visualisation des acides nucléiques polymérisés est ensuite réalisée par un marquage employant un intercalant de l'ADN qui sera révélé par une exposition aux UV. Par la suite la RT-PCR puis la qPCR (aussi nommée PCR en temps réel) ont été développées, permettant respectivement la détection des génomes ARN

après transcription en ADN complémentaire et la quantification de l'ADN polymérisé à chaque cycle et par extension des génomes présents initialement dans l'échantillon.

b. Les virus entériques

La mise au point, au cours des années 1990, de méthodes de détection et de quantification des virus plus précises et robustes a permis le développement de la virologie environnementale en tant que discipline de recherche à part entière avec par exemple la mise au point des techniques de biologie moléculaire qui permettent de passer outre les limites de la détection par culture cellulaire (Metcalf *et al.*, 1995). La virologie environnementale a pour objectif principal de décrire et comprendre les processus impliqués dans l'arrivée des pathogènes viraux dans l'environnement et leur devenir au sein de celui-ci afin de mieux saisir le risque sanitaire, d'une infection de l'Homme après avoir séjourné dans l'environnement. Parmi les pathogènes étudiés par cette discipline, les virus entériques de l'Homme sont particulièrement suivis car ils sont connus pour être une source importante d'épidémies à travers le monde, qu'elles soient d'origine alimentaire ou hydrique (Kirk *et al.*, 2015; La Rosa *et al.*, 2012; WHO, 2015). Leur importance est telle qu'ils sont actuellement inclus dans la *Contaminant Candidate List* de l'Agence Américaine de Protection de l'Environnement (US EPA) qui recense les contaminants d'importance majeure à suivre dans les eaux de boisson mais ne faisant pas encore l'objet de réglementations (US EPA, 2014) et dans celle proposée par l'American Water Works Association (Hoffman *et al.*, 2009).

Les virus entériques de l'Homme sont des virus pathogènes de l'être humain, qui en est de fait le principal réservoir. Le tropisme essentiellement gastro-intestinal de ces virus a pour conséquence une prédominance des maladies touchant le tractus digestif. Les symptômes associés sont en très grande majorité des gastro-entérites principalement aigües d'intensité variable et des hépatites. D'autres symptômes tels que des hépatites, des méningites, accompagnées de conjonctivites ou de fièvres sont rapportés (La Rosa *et al.*, 2012; Le Guyader *et al.*, 2014).

Les gastroentérites correspondent selon Majowicz et al. (2008) à au moins trois selles non-formées ou à au moins un épisode de vomissement durant une période de 24 heures, en excluant les cas associés aux femmes enceintes ou à des patients atteints de cancers ou de maladies chroniques présentant ce type de symptômes. D'autres symptômes peuvent apparaitre, tels que des douleurs abdominales, de la déshydratation, des vertiges ou des céphalées. Il s'agit d'affections du petit et du gros intestin présentes, ou du foie. La dernière étude de l'OMS menée sur le sujet a conclu que pour la seule année 2010 il était survenu près de 1,8 milliards de cas de gastroentérites d'origine alimentaire seulement (Kirk *et al.*, 2015; Pires *et al.*, 2015; WHO, 2015). Parmi celles-ci les virus seraient responsables de 36% des gastroentérites aiguës dans le monde (550 millions de cas par année), et jusque 67% des cas en Europe (Kirk *et al.*, 2015). Des variations géographiques sont visibles avec une plus forte

prévalence des infections bactériennes et parasitaires sur les infections virales dans les régions de l'Afrique et de l'Asie du Sud-Est (Kirk *et al.*, 2015; Pires *et al.*, 2015).

Les virus entériques de l'Homme sont majoritairement représentés par les pathogènes suivants (**Tableau I-1**) :

- Les *Caliciviridae* constituent une famille de virus nus (27-42 nm) à ARN simple brin incluant les norovirus (NoV). Ce sont les principaux agents étiologiques des maladies d'origine alimentaire ou hydrique à l'heure actuelle. Aussi nommés « Winter vomiting disease viruses », les norovirus sont les principaux responsables des épidémies annuelles de gastroentérites qui surviennent durant l'hiver dans l'hémisphère Nord (Ahmed *et al.*, 2013; Costantini *et al.*, 2016; Jagai *et al.*, 2012; Pivette *et al.*, 2014; Prevost *et al.*, 2015b; Reynolds *et al.*, 2008; Rivière *et al.*, 2017). Il est à noter que le virus est présent dans le monde entier mais avec une saisonnalité moins marquée en Afrique (Mans *et al.*, 2016). Les génogroupes prédominants dans les épidémies actuelles sont les génogroupes I et II (van Beek *et al.*, 2013) . La mortalité globale associée à une infection à norovirus est évaluée à environ 30% des décès associés à une maladie diarrhéique (Kirk *et al.*, 2015) et son incidence est évaluée entre 0,19 et 0,40 décès pour 10 000 individus aux U.S.A. et aux Pays-Bas (Hall *et al.*, 2012; Verhoef *et al.*, 2013). Les norovirus peuvent infecter toute la population quel qu'en soit l'âge ou l'origine (Bok & Green, 2012; Desai *et al.*, 2012; Green, 2014; Reynolds *et al.*, 2008; Ruzante *et al.*, 2011). Il n'existe pas de vaccin efficace pour l'heure. Les norovirus humains sont très difficilement cultivables (Ettayebi *et al.*, 2016) .
- Les Adénovirus (AdV) sont des virus nus (50-60 nm) à ADN double brin de la famille des *Adenoviridae* et se répartissent en 7 sous-groupes capables de causer des infections respiratoires ou intestinales avec comme résultantes pneumonies, conjonctivites, gastroentérites modérées ou myocardites (Lynch & Kajon, 2016; Skalka *et al.*, 2015). Il n'existe pas de réelle saisonnalité au regard des infections à adénovirus mais il apparait que 80% de la population mondiale est supposée avoir déjà été infectée (Skalka *et al.*, 2015). Les adénovirus sont connus pour être l'une des causes majeures de décès chez les individus immunodéprimés et/ou transplantés avec un taux de létalité situé entre 18% et 60% (Reynolds *et al.*, 2008).
- Les Rotavirus (RV) sont des virus nus (80 nm) à ARN double brin (famille *Reoviridae*) et connus pour être responsables de la moitié des gastroentérites virales de l'enfant de moins de 2 ans (Mukhopadhya *et al.*, 2013). Présents sur toute la surface du globe, ils présentent une légère saisonnalité avec une baisse des infections durant la période estivale (Patel *et al.*, 2013). Du fait de la population cible, le rotavirus présente une morbidité importante. Il est aujourd'hui admis que ce virus est responsable d'environ 50% des cas de gastroentérites aigües chez l'enfant et de de 215 000 décès par an, dont 50% seraient dus aux déshydrations liées aux diarrhées dans les pays les plus pauvres (Skalka *et al.*, 2015; Tate *et al.*, 2016).

- Les Entérovirus (EV) sont des virus nus (27-30 nm) de la famille des *Picornaviridae* et à ARN simple brin. Contrairement à ce que sous-entend leur nom, le tropisme des Entérovirus n'est pas spécifiquement entérique mais peut aussi être respiratoire dans le cas de l'Entérovirus 68. Parmi les Entérovirus à tropisme intestinal se trouvent les Poliovirus, les Echovirus et les Coxsackievirus A et B responsables respectivement de poliomyélites, éruptions cutanées et syndromes pieds-mains-bouche. Les diarrhées, conjonctivites et infections respiratoires sont aussi rapportées (Skalka *et al.*, 2015). Le virus est présent sur tout le globe avec une saisonnalité estivale contrairement à la plupart des autres virus entériques (Dowell, 2001). Les Entérovirus sont capables d'infecter toute la population avec un risque accru concernant les jeunes enfants notamment en ce qui concerne l'Entérovirus 71, agent étiologique du syndrome pieds-mains-bouche.
- Le virus de l'hépatite A (VHA) est aussi un virus nu à ARN simple brin de la famille des *Picornaviridae* mais appartenant au genre *Hepatovirus* (Pintó *et al.*, 2010). Celui-ci se distingue à la fois par son tropisme hépatique mais aussi par sa plus grande résistance aux contraintes environnementales telles que les hautes températures, les pH acides ou les traitements d'inactivation du virus (Martin & Lemon, 2006). Comme l'indique son nom, le virus de l'hépatite A est capable d'induire chez le patient infecté une hépatite A dont les symptômes (diarrhées, fatigue, fièvre, céphalées …) sont peu spécifiques à l'exception des ictères (coloration jaune de la peau ou des muqueuses). La saisonnalité du virus est connue pour être hivernale comme pour de nombreux autres virus entériques (Gharbi-Khelifi *et al.*, 2007). Le virus touche toute la population avec un risque de développer une hépatite fulminante mortelle à 70% chez moins de 1% des patients (Taylor *et al.*, 2006). En dehors de ces cas particuliers, le virus de l'hépatite A est la deuxième cause de décès par maladie entérique invasive selon l'OMS avec 93961 décès dans le monde en 2010 (Kirk *et al.*, 2015). Il est à noter que le virus est presque absent du territoire français, où la très grande majorité des cas qui y surviennent sont des cas rapportés de régions où le virus est endémique comme l'Afrique du Nord, l'Asie du Sud-Est, l'Amérique du Sud ou l'Europe de l'Est (Jacobsen & Wiersma, 2010). La séroprévalence y atteint 90% chez les enfants de moins de 10 ans, pour lesquels l'infection est majoritairement asymptomatique contrairement aux individus de plus de 50 ans chez lesquels le risque de développer une hépatite fulminante sont accrus (Jacobsen & Wiersma, 2010). Le taux de mortalité évolue proportionnellement à l'âge du patient avec un taux de 0.23% chez les moins de 30 ans, de 0,3 à 0,6% jusque 49 ans puis de 1,8 à 2,1% par la suite,

Du fait de la fragilité de l'enveloppe virale des virus enveloppés face aux contraintes environnementales et aux traitements désinfectants, les virus nus constituent la plus grande partie des virus entériques. Néanmoins quelques virus enveloppés peuvent être détectés dans les selles de patients atteints de gastroentérites comme les Coronavirus (Jevšnik *et al.*, 2013).

Tableau I-1 : Description des caractéristiques épidémiologiques des principaux virus entériques retrouvés en milieu hydrique (adapté de Le Guyader et al. (2014))

Nom	Adénovirus	Norovirus	Entérovirus	Rotavirus	Virus de l'hépatite A
Pathologies	Pneumonie Gastroentérite Cystite Conjonctivite Myocardite	Gastroentérite	Pieds – Mains – Bouches Gastroentérite Paralysie Méningite Conjonctivite	Gastroentérite	Hépatite
Vaccin	Oui	Aucun	Pour Poliovirus	Oui	Oui
Saisonnalité	Toute l'année	Hiver	Été	Hiver	Hiver
Endémicité	Mondiale	Mondiale	Mondiale (sauf Poliovirus)	Mondiale	
Population cible	Jeunes enfants	Tout âge	Tout âge	Jeunes enfants	Tout âge

À l'exception des norovirus, la plupart des virus entériques peuvent être isolés sur culture cellulaire. Pour parvenir à étudier facilement l'évolution de l'infectivité de ces virus dans le cadre d'expériences, les chercheurs utilisent régulièrement des virus modèles. Les virus modèles sont des virus qui partagent une partie de leurs caractéristiques (tout ou partie des critères suivants : taille, point isoélectrique, tropisme, récepteurs cibles, résistance aux conditions environnementales) avec un virus dangereux et/ou difficilement cultivable mais présentent l'avantage de pouvoir être produits en grandes quantités et sans difficulté majeure. Les conclusions de l'étude de ce modèle sont alors supposés transposables au virus qu'il « représente ». Les bactériophages MS2, λ et ΦX174 sont communément considérés comme les virus modèles de choix pour l'étude des virus entériques (Leclerc *et al.*, 2000) car ils sont très simples à produire (une bactérie contaminée peut libérer 50 à 100 bactériophages lors de sa lyse) et présentent une nucléocapside nue de symétrie icosaédrique et une résistance au pH acides proches de celle des virus entériques (Bae & Schwab, 2008). Dans le cadre plus spécifique de l'étude des norovirus l'un des virus modèles de choix est le norovirus murin (MNV) qui est un agent de la gastroentérite chez la souris de la famille des *Caliciviridae* (Bae & Schwab, 2008; Belliot *et al.*, 2008; Wobus *et al.*, 2006). D'autres modèles existent tels que le calicivirus félin (FCV) (Bae & Schwab, 2008; Cannon *et al.*, 2006) et le virus Tulane (Hirneisen & Kniel, 2013b), *Caliciviridae* infectant respectivement le tractus respiratoire des félins et le tractus digestif chez le macaque rhésus (Arthur & Gibson, 2016). Concernant le virus de l'hépatite A, son principal modèle viral est le mengovirus murin.

Tableau I-2: Taux d'atteinte médian des infections (et décès) pour 100 000 habitants associées aux gastroentérites selon l'agent étiologique et la région du monde (tiré de Kirk et al. (2015))

Pathogène	Europe	Afrique	Amérique	Moyen-Orient	Asie du Sud-Est	Pacifique Ouest	Global
Norovirus	1652 (0,05)	1749 (1)	2491 (0,1)	2796 (0,4)	841 (1)	1305 (0,05)	1814 (0,5)
Campylobacter spp	522 (0,05)	2221 (0,8)	1389 (0,07)	1873 (1)	1152 (0,4)	876 (0,04)	1390 (0,3)
Cryptosporidium spp	21 (0,003)	205 (0,2)	114 (0,007)	346 (0,04)	78 (0,09)	32 (0,003)	125 (0,05)
ETEC	6 (0)	982 (1)	1281 (0,05)	4971 (0,4)	1075 (0,6)	555 (0,04)	1257 (0,4)
Shigatoxin producing *E. coli*	18 (0,003)	5 (0)	16 (0,0004)	65 (0,002)	19 (0,002)	3 (0)	17 (0,002)
V. cholerae	0,03 (0)	43 (2)	0,02 (0)	9 (0,3)	17 (0,4)	0,2 (0,002)	11 (0,4)

La prévalence importante des virus entériques (**Tableau I-2**) dans le monde est profondément associée à leurs voies de contamination et aux symptômes qu'ils induisent chez le patient symptomatique, de la prophylaxie et de leur résistance aux contraintes environnementales. En effet, ces virus sont en mesure de demeurer infectieux durant plusieurs mois dans l'eau et sur certaines surfaces (Ogorzaly *et al.*, 2010; Yeargin *et al.*, 2016) en fonction des conditions de température (Bertrand *et al.*, 2012; Kim *et al.*, 2012), d'humidité (Colas de la Noue *et al.*, 2014; Kim *et al.*, 2012), de pH et d'ensoleillement ainsi que de la composition chimique de l'environnement.

Au-delà de cette résistance à l'environnement, il ne suffit la plupart du temps que d'une dizaine de particules virales pour infecter l'être humain, *via* la voie oro-fécale, et déclencher la maladie (Atmar *et al.*, 2014). Dès l'infection, le virus va se multiplier dans le tractus digestif de l'hôte puis se trouver excrété lors des émissions de selles et des vomissements par le patient à des concentrations pouvant aller jusque 10^{11} virus.g^{-1} de selles dans le cas des norovirus (Atmar *et al.*, 2008; Teunis *et al.*, 2015). Deux points sont à noter dans le cas de ce type de virus : le premier est que l'excrétion des virus ne se limite pas à la période symptomatique de la maladie. Par exemple le rotavirus, est encore excrété au moins 21 jours après la fin de la GEA (Mukhopadhya *et al.*, 2013), et le norovirus l'est encore pendant 20 à 40 jours (Atmar *et al.*, 2008; Newman *et al.*, 2016a; Sabrià *et al.*, 2016; Teunis *et al.*, 2015). Un autre point important est que pour tous les virus entériques, il existe une population asymptomatique, c'est-à-dire qui n'exprimera pas la maladie (elle est évaluée à

30% de la population infectée pour le norovirus GII (Teunis *et al.*, 2015)). Néanmoins cette population est toujours source de pollution virale car elle excrète les virus dans les même quantités que les patients symptomatiques, exception faite du rotavirus, mais ne prendra pas de mesures de mise à l'écart ce qui amènera à la contamination de l'environnement et de nombreuses surfaces (Franck *et al.*, 2015; Lopman *et al.*, 2014; Newman *et al.*, 2016a; Phillips *et al.*, 2010; Teunis *et al.*, 2015).

3. Devenir environnemental des virus (état des connaissances)

a. Devenir dans les eaux usées

La contamination virale des cours d'eau et son lien avec les épidémies virales d'origine hydrique est maintenant clairement établi (Prüss-Ustün *et al.*, 2014) avec une concentration pouvant atteindre de 10^4 à 10^6 gc.L^{-1} pour les norovirus et les adénovirus, respectivement (D'Ugo *et al.*, 2016; Prevost *et al.*, 2015b; Sinclair *et al.*, 2009; Vergara *et al.*, 2016; Wyn-Jones *et al.*, 2011), et avec des variations saisonnières autant en termes de quantité que de diversité (Brinkman *et al.*, 2017; Prevost *et al.*, 2015a, 2015b). Ces évolutions dans le temps sont le reflet des rejets d'eaux usées, principalement domestiques, des excrétas viraux qu'elles transportent et par conséquent de l'état de santé de la population. Il est à noter qu'en 2010 la part de la population mondiale ayant accès à des sanitaires corrects, c'est-à-dire reliés à un réseau d'assainissement amenant à un traitement des eaux usées avant leur rejet dans l'environnement, était estimée à 40% (Baum *et al.*, 2013). Cette proportion varie en particulier selon le niveau d'industrialisation des pays (WHO & UNICEF, 2015). Ainsi pour des pays d'Europe occidentale tels que la France, le Royaume-Uni ou l'Allemagne cette proportion atteignait 74, 95 et 95%, respectivement, en 2010 (Baum *et al.*, 2013), la France étant désormais à 95,5% (Observatoire national des services d'eau et d'assainissement, 2013). D'autres pays européens ont vu augmenter de façon significative ce taux entre 1990 et 2010, par exemple Chypre (de 1,4 à 39,7%) et la Grèce (de 16 à 78%). Concernant les pays en voie d'industrialisation, les taux sont beaucoup plus faibles. En effet, là où l'on considère que 71% des eaux usées municipales et industrielles sont traitées en Europe, seuls 51% le sont au Moyen Orient et en Afrique du Nord, ce taux tombant à 20% en Amérique latine (World Water Assessment Programme, 2017). Parallèlement à cela, des systèmes d'assainissement individuels, de type fosse septique, sont accessibles à une partie de la population, évaluée à 17% pour la France en 2008 (Ministère de la transition écologique et solidaire, 2011).

Les concentrations en virus entériques pathogènes de l'Homme dans les égouts varient en fonction de plusieurs facteurs :

- La charge virale excrétée par la population dans le réseau d'assainissement, sachant que dans le seul cas du norovirus un individu malade peut excréter jusque 10^{11} virus.g^{-1} de selles durant plusieurs semaines après l'infection (**Figure I.5**), qu'il y ai eu ou non symptômes (Teunis *et al.*, 2015) ;

- Les fuites dans le réseau qui peuvent atteindre 10% du volume total d'eaux usées circulantes (Roehrdanz *et al.*, 2017) et qui peuvent surtout contaminer les réseaux d'eau potable à proximité, les nappes d'eau et les sources d'eau associées (Bradbury *et al.*, 2013; Gotkowitz *et al.*, 2016; Roehrdanz *et al.*, 2017). Le nombre et l'importance de ces fuites est profondément lié aux matériaux constituant les canalisations de même que l'âge de ces dernières (Gotkowitz *et al.*, 2016). Par ailleurs, ce sont aussi l'âge et le matériau des canalisations qui vont influencer la survie des virus durant leur transit, notamment en favorisant l'apparition de conditions réductrices et en libérant des oxydes de fer lors de l'usure du béton constituant les canalisations (Hvitved-Jacobsen *et al.*, 2013) ;

- Enfin, les particules virales peuvent être diluées lorsque des précipitations aboutissent au mélange d'eaux usées domestiques et d'eaux de ruissellement dans les réseaux unitaires. Un effet secondaire associé aux fortes précipitations est la mise en place de dérivations (nommées « by-pass ») dans le réseau permettant de dévier le trop-plein d'eaux usées arrivant à la station vers l'environnement. Ce système permet de maintenir l'efficacité du traitement en station mais entraîne une pollution environnementale supplémentaire en rejetant les eaux usées brutes, diluées, directement dans la nature.

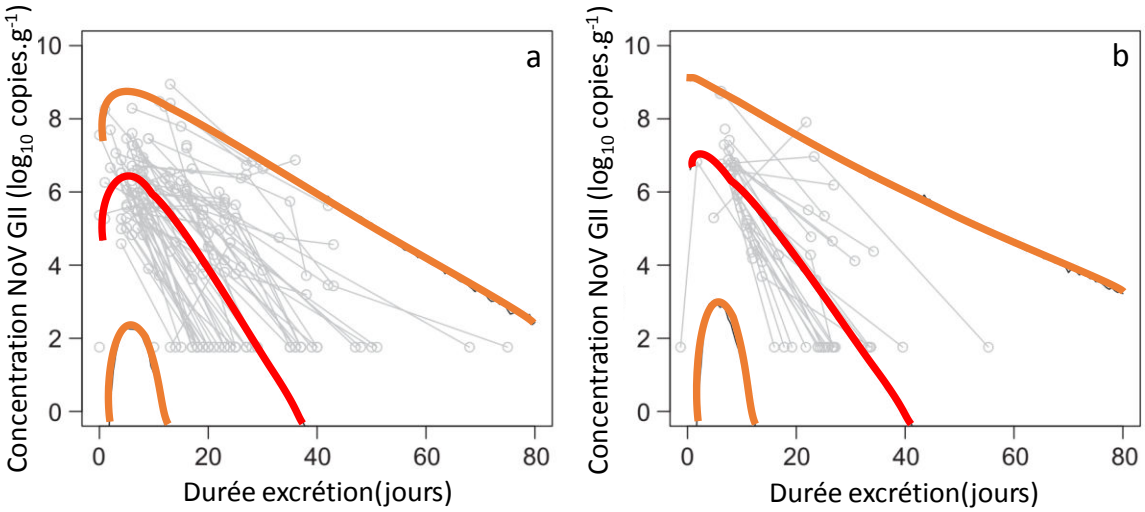


Figure I.5: Modélisation d'excrétion virale durant une infection à norovirus GII. Les courbes rouge et orange décrivent respectivement la médiane et les intervalles à 95% de l'excrétion. a) Cas d'un patient symptomatique. b) Cas d'un patient asymptomatique (Teunis *et al.*, 2015).

En station d'épuration des eaux usées, les traitements réalisés en station ont pour objectif principal de réduire la charge en matières organiques en suspension dans les eaux usées brutes. Le premier traitement, dit primaire, permet le retrait des matières solides grossières et d'une partie des graisses présentes dans les eaux usées brutes et ne permet qu'un très faible abattement bactérien et viral (Campos *et al.*, 2016). Ce traitement est réalisé par dégrillage, sédimentation puis déshuilage et permet la bonne réalisation des traitements suivants. Le deuxième traitement permet de réaliser la plus grande partie de l'abattement en matières organiques et correspond très souvent au dernier traitement avant le rejet des eaux usées. Celui-ci peut être réalisé de plusieurs façons et notamment *via* un traitement biologique seul (boues activées) ou couplé à un traitement d'ultrafiltration dans le cas des bioréacteurs à membrane. En ce qui concerne les petites villes ou villages, le choix du traitement se porte souvent sur un lagunage du fait de son rapport coût-efficacité très intéressant à cette échelle. Enfin certaines stations d'épuration réalisent un traitement, dit tertiaire, à base de traitements physiques (diverses filtrations, UV), ou de traitements chimiques (chlore actif, ozone, acide peracétique), voire par lagunage, afin d'atteindre un abattement plus important en pathogènes et/ou en micropolluants organiques. Les traitements de filtrations peuvent aller d'une filtration sur sable à une osmose inverse. L'un des intérêts de cette dernière (classiquement utilisée pour dessaler les eaux de mer et les eaux saumâtres) pour des eaux usées est d'éliminer leur charge en sels et par mélange à des eaux usées trop chargées d'aboutir à des salinités compatibles avec le type de réutilisation visée des eaux usées (notamment l'irrigation agricole).

Plusieurs études ont cherché à évaluer l'action de ces traitements sur l'abattement viral avec la réalisation de travaux de terrain (Katayama *et al.*, 2008; La Rosa *et al.*, 2010; Purnell *et al.*, 2016; Sima *et al.*, 2011), de revues (Campos *et al.*, 2016; Verbyla & Mihelcic, 2015) et de méta-analyses (Pouillot *et al.*, 2015; Sano *et al.*, 2016) mais aussi en confrontant un modèle aux données expérimentales (Haun *et al.*, 2014). Malheureusement, la plupart des études ne prennent pas en compte certains paramètres pouvant affecter l'abattement viral en station tels que l'aération des bassins de boues activées, la taille des pores des bioréacteurs à membrane, la nature des composés employés pour provoquer la floculation des boues ; ces derniers pouvant entraîner la co-précipitation des virus (Haun *et al.*, 2014) ; les traitements de désinfection tels que l'acide peracétique (Dunkin *et al.*, 2017; Mattle *et al.*, 2011) ou le temps de séjour des eaux en station (Katayama *et al.*, 2008). De plus certains facteurs environnementaux nécessitent d'être pris en compte tels que la température des eaux, le rayonnement solaire ou l'agrégation des virus (entre eux ou sur des particules) qui va fortement affecter leur résistance aux traitements (Da Silva *et al.*, 2008; Mattle *et al.*, 2011; Mattle & Kohn, 2012). Le dernier facteur à prendre en compte concerne le virus lui-même, sa résistance aux pressions environnementales et aux traitements en station variant très fortement selon l'espèce et la souche virale étudiée dans le cas des norovirus notamment avec une meilleure résistance aux traitements du génogroupe GI vis-à-vis du GII (**Tableau I-3**), quand bien même ce dernier est le plus souvent représenté dans les eaux usées brutes (da Silva *et al.*, 2007; Petrinca *et al.*, 2009; Sigstam *et al.*, 2013; Wigginton & Kohn, 2012).

La majorité des stations d'épuration a recours aux traitements primaires classiques (dégrillage et sédimentation) suivis par un traitement par boues activées qui permettent d'atteindre un abattement des norovirus situé entre 1 et 3 $\log_{10}$ en moyenne (Campos *et al.*, 2016; La Rosa *et al.*, 2010; Sima *et al.*, 2011). Dans le cas d'un traitement secondaire par bioréacteur à membrane, l'abattement réalisé se révèle plus important avec des valeurs pouvant atteindre 4,7 $\log_{10}$ (Simmons & Xagoraraki, 2011). De même (La Rosa *et al.*, 2010) ont calculé que, dans le cas de 5 stations d'épuration autour de Rome employant la méthode des boues activées, des abattements en log10 de 0,11 et 0,20 peuvent être atteints en ce qui concerne les norovirus GI et GII, respectivement, 0,46 et 0,11 pour les EV et les adénovirus et près de 2 en ce qui concerne les *E. coli* et Entérocoques.

Tableau I-3: Abattements moyens sur les concentrations en Norovirus réalisés en station de traitement des eaux usées en fonction des technologies d'assainissement utilisées.

Traitement		**Abattement viral ($\log_{10}$)**		**Références**
Secondaire	**Tertiaire**	**NoV GI**	**NoV GII**	
Boues activées	-	0,7	1,2	Nordgren et al. (2009)
		2,2	3,1	Pérez-Sautu et al. (2012)
		0,8	0,92	Flannery et al. (2012)
		0,11	0,20	La Rosa et al. (2010)
		1,48	1,35	Sano et al. (2016)
		1,30	1,69	Campos et al. (2016)
	UV	2,31	2,60	Campos et al. (2016)
Bioréacteur à membrane	-	0,9	-	Sima et al. (2011)
		-	4,7	Simmons et Xagoraraki (2011)
		-	3.35	Sano et al. (2016)
		2,3	2,3	Purnell et al. (2016)
		1,80	2,40	Campos et al. (2016)
	UV	N.D.	2,68	Campos et al. (2016)
Lit bactérien		1,71	1,93	Campos et al. (2016)

Du fait des limites des techniques actuelles de détection et de quantification des virus entériques, autant en termes de temps et de moyens, de nombreuses études ont cherché à évaluer la pollution virale dans les eaux de station et de rivière par le biais d'organismes dits indicateurs. Ces organismes doivent répondre à plusieurs exigences : leur excrétion doit se dérouler concomitamment et en quantités proportionnelles à celles des virus entériques de même que leur résistance aux traitements en station doit être équivalente. Par conséquent, la bactérie *E. coli* représente l'organisme indicateur de référence pour le suivi des pollutions fécales bactériennes. L'abattement en station des bactériophages est aussi suivi dans le même but, en particulier à cause de leur plus grande résistance aux traitements en station (Fout *et al.*, 2017; Ministère de la Santé et des Sports, 2010). Néanmoins la majeure partie des études

s'accorde à dire que le suivi de ces indicateurs biologiques est peu pertinent (Payment & Locas, 2011).

b. Devenir dans les eaux douces et souterraines

Les virus se révèlent la plupart du temps plus difficiles à détecter dans les eaux environnementales que dans les eaux usées, ce qui est lié à de nombreux facteurs. Le premier est l'effet de la dilution des eaux usées lors de leur rejet dans l'environnement. En effet, la plupart des stations de traitement des eaux usées vont réaliser le rejet des eaux traitées directement dans les eaux de surface en comptant sur la dilution pour encore réduire la pollution liée aux rejets. Ce phénomène fonctionne très bien dans le cas de rejets dans les rivières de grande taille ou les zones côtières de par les volumes d'eau très élevés. Un autre facteur participant à la difficulté de la détection environnementale des virus entériques est celui de la survie des virus dans les rivières ou les nappes. En effet, cette survie est fortement dépendante des paramètres physico-chimiques du milieu. Comme décrit par Gerba (2007) ainsi que Kotwal & Cannon (2014) ces facteurs sont la température, la lumière, les sels et matières organiques présentes dans l'eau, les sédiments transportés, les interfaces entre l'eau et l'air ainsi que les facteurs biologiques. La température de l'eau apparait comme le principal facteur pouvant influencer la survie des virus en solution (Bertrand *et al.*, 2012). De nombreuses études ont pu démontrer l'accroissement de la survie des virus avec la baisse des températures notamment dans le cas du poliovirus (Bae & Schwab, 2008) ou du virus de l'hépatite A notamment (Dalziel *et al.*, 2016). Ceci s'explique par le fait que les températures plus faibles réduisent les risques de dénaturation des capsides virales ainsi que des génomes associés. Concernant l'influence de la lumière, celle-ci est associée au pouvoir mutagène des rayons UVC (254 nm) sur le génome viral, induisant l'apparition de mutation délétères et réduisant l'infectivité du virus sur les premiers centimètres de la colonne d'eau. Les sels, matières organiques et sédiments présents dans les eaux ont pour effet connu de protéger certains virus des effets de températures élevées, en particulier les PV dans le cas des eaux marines (Liew & Gerba, 1980). Le facteur biologique est quant à lui connu pour avoir un effet majoritairement négatif sur la survie virale en milieu aquatique même si quelques études ont pu révéler un effet plus modéré dans le cas particulier des biofilms bactériens (Heistad *et al.*, 2009; Skraber *et al.*, 2005).

Les virus rejetés dans l'environnement avec les eaux usées vont néanmoins être en mesure, de par leur taille infra micrométrique et leur nombre conséquent, de contaminer à des concentrations non-négligeables tous les compartiments de l'eau existants, de la rivière (Prevost *et al.*, 2015b) à l'aquifère (Murphy *et al.*, 2017) en passant par les eaux marines (Gibbons *et al.*, 2010; Haramoto *et al.*, 2006; Vergara *et al.*, 2016; Wyn-Jones *et al.*, 2011) Par ailleurs, comme indiqué précédemment, les variations saisonnières des concentrations et diversités des virus présents dans les eaux traduit l'état de santé général des populations, voire le caractère endémique de certaines infections dans certaines régions du monde. Il apparait

ainsi qu'en Europe les lacs et rivières sont majoritairement contaminés par des norovirus et des adénovirus, respectivement à hauteur de 10^3 à 10^4 gc.L^{-1} et 10^3 à 10^6 gc.L^{-1} (D'Ugo *et al.*, 2016; Vergara *et al.*, 2016; Wyn-Jones *et al.*, 2011) avec de très rares détections de virus de l'hépatite A (D'Ugo *et al.*, 2016). Dans le cas de l'Amérique latine, le constat se révèle différent avec des détections plus fréquentes du virus de l'hépatite A (environ 92% des échantillons), endémique de la région et détecté à des concentrations pouvant dépasser 10^3 gc.L^{-1} (De Paula *et al.*, 2007; Villar *et al.*, 2007). Ce constat est aussi valable à l'échelle urbaine (Hewitt *et al.*, 2011) comme dans le cas de la Seine et de ses effluents où les ratios entre les diverses populations de virus entériques évoluent en fonction des saisons et en accord avec les espèces circulantes dans la population parisienne (Prevost *et al.*, 2015b). Le même résultat a pu être atteint lors d'un suivi des virus entériques dans une rivière de Caracas (Venezuela) contaminée par les eaux usées (Rodríguez-Díaz *et al.*, 2009). Dans certains cas les concentrations peuvent être équivalentes à celles retrouvées dans les eaux usées traitées, soulignant de ce fait une contribution très importante des eaux usées traitées au débit du cours d'eau ou la possibilité de rejets d'eaux usées non-traitées issues par exemples d'assainissements non-collectifs défaillants (Berendes *et al.*, 2017) ou d'incidents survenus en station lors d'un traitement.

En dehors des rivières, les autres compartiments de l'eau sont considérés comme étant contaminés par des virus entériques, mais à diverses concentrations. Les aquifères et nappes alluviales, souvent exploitées pour le captage d'eau potable, peuvent se trouver face à un risque de contamination virale (Fout *et al.*, 2017) ou le sont déjà depuis très longtemps. Murphy et al (2017) ont par exemple étudié les sources d'épidémies de gastroentérites survenues dans le monde entre 1948 et 2015 et sont parvenu à établir une étiologie virale dans 40% des 92 épidémies retenues pour l'étude. Lee et al (2011) ont évalué entre 18% et 28% la proportion des réserves d'eau souterraine de Séoul (Corée du Sud) contaminées par des norovirus, respectivement en période estivale ou hivernale. De leur côté Jung et al (2011) ont détecté des Norovirus dans 18% des échantillons, prélevés dans des puits à travers le pays, ainsi que des entérovirus et des Adénovirus. Aux États-Unis, une étude menée sur trois puits de captage d'eau potable a révélé la contamination de deux d'entre eux par des entérovirus (Borchardt *et al.*, 2007). Une étude précédente a révélé la présence d'entérovirus dans 15% des puits testés, de même que celle de rotavirus (14%) et de virus de l'hépatite A (7%) (Abbaszadegan *et al.*, 2003). Par ailleurs la survie de l'adénovirus dans des eaux souterraines, conservées à l'obscurité, a révélé un abattement de l'infectivité du virus de 2,4 $\log_{10}$ en quatre mois lorsqu'il est stocké à 20°C et un maintien de son ADN, qui reste détectable par PCR, durant près de 400 jours (Ogorzaly *et al.*, 2010). Le norovirus GI reste en mesure de provoquer l'apparition d'une gastroentérite aigüe après 61 jours de conservation à l'obscurité et sa détection reste possible après 3 ans de stockage (Seitz *et al.*, 2011). Le constat selon lequel les eaux souterraines pouvaient être considérées comme saines pour la consommation humaine a dû être révisé de même que la réglementation américaine pour inclure le suivi de ces virus (Hoffman *et al.*, 2009; US EPA, 2014).

Le cas des eaux estuariennes est plus particulier en premier lieu à cause du fait que ces eaux sont particulièrement riches en sels minéraux, matières organiques et sédiments mais aussi en raison d'une microflore à la fois très diversifiée et abondante et d'un ensoleillement important. De fait il est possible de détecter dans les eaux côtières des entérovirus et adénovirus à des concentrations respectives de l'ordre de 10^3 gc.L^{-1} et 10^8 gc.L^{-1} (Hassard *et al.*, 2016). Hernandez-Morga et al (2009) ont, lors d'une campagne de prélèvements réalisée dans un estuaire situé dans le golfe de Californie, récolté 40 échantillons d'eau dont 80% se sont révélés contaminés par le virus de l'hépatite A et 70% par le norovirus GII. À titre de comparaison, *E. coli* n'a été détectée que dans 34% des échantillons. De la même façon, Haramoto et al (2006) sont parvenus à détecter des particules d'adénovirus dans plus de 80% des échantillons d'eau de mer prélevés sur une campagne de 2 mois, avec la mise en évidence de l'influence exercée par les précipitations sur ces concentrations. Néanmoins les particules virales présentes dans ces eaux ne restent que très rarement en suspension. En effet, les conditions physico-chimiques du milieu et les activités biologiques qui s'y déroulent vont avoir pour effet de favoriser le stockage des virus, principalement de deux manières : par adsorption sur des sédiments (Hassard *et al.*, 2016) ou par bioaccumulation dans les organismes filtreurs comme les Mollusques bivalves (Gentry *et al.*, 2009; Mojica & Brussaard, 2014). De fait, une fois adsorbés sur les sédiments, ceux-ci se trouvent mieux protégés face aux conditions environnementales et, dans le cas de tempêtes, peuvent même contaminer les eaux marines de façon plus importante que lors d'un rejet d'eaux usées *via* la remise en suspension des sédiments (Dorner *et al.*, 2006; Pommepuy *et al.*, 2005; Rao *et al.*, 1986). Par ailleurs la bioaccumulation des particules virales dans les tissus des mollusques bivalves semble accroître leur survie et engendre de fait un nouveau problème de santé publique liée à la consommation de ces produits, particulièrement en ce qui concerne les norovirus et le virus de l'hépatite A (Iaconelli *et al.*, 2015; Westrell *et al.*, 2010)

c. Devenir dans le sol

Les virus entériques peuvent atteindre et contaminer les sols lors d'événements de pollution, par exemple lors de fuites dans les réseaux de canalisation d'eaux usées (Hunt & Johnson, 2017; Roehrdanz *et al.*, 2017), ou à l'occasion d'irrigations avec des eaux contaminées, douces comme usées (Girardin *et al.*, 2016). Au sol ces virus peuvent soit circuler, en solution, en même temps que les eaux et atteindre des aquifères (Murphy *et al.*, 2017) ou être absorbés par les racines des végétaux et contaminer les parties comestibles (DiCaprio *et al.*, 2015; Hirneisen & Kniel, 2013a, 2013c). Le devenir des virus dans les sols va dépendre de leur transport, de leur immobilisation et de leur inactivation (Chattopadhyay & Puls, 2000; Dowd *et al.*, 1998; Seitz *et al.*, 2011; Zhao *et al.*, 2008). Ces trois phénomènes sont d'ailleurs interdépendants et les virus peuvent circuler à la fois sous forme de particules libres (seules ou agrégées) ou immobilisés sur des colloïdes circulants (Katzourakis & Chrysikopoulos, 2015; Syngouna & Chrysikopoulos, 2010). De plus une immobilisation dans

certaines matrices peut avoir pour effet de rendre un virus plus résistant aux contraintes environnementales, comme dans le cas des biofilms bactériens (Heistad *et al.*, 2009), ou peut les inactiver beaucoup plus rapidement, en particulier dans le cas d'une adsorption sur des oxydes métalliques (Murray & Laband, 1979; Park & Kim, 2015; Warnes *et al.*, 2015; Zhuang & Jin, 2008).

L'immobilisation des virus va être médiée par plusieurs facteurs :

- Les caractéristiques physiques du virus font influencer ses interactions avec son milieu. En effet l'espèce virale et la souche étudiée peuvent faire varier la composition protéique de l'enveloppe ou de la nucléocapside des virus ainsi que la conformation de ces dernières (Goodridge *et al.*, 2004; Michen & Graule, 2010). Cette diversité va avoir pour effet de modifier, entre autres, le point isoélectrique du virus (Michen & Graule, 2010) et ses interactions électrostatiques avec des surfaces chargées ou la taille du virion et ses interactions stériques au sein du sol (Dowd *et al.*, 1998; Taylor *et al.*, 1981). Par exemple les échovirus humains de type 1 voient leur point isoélectrique varier entre 4,0 et 6,4 en fonction de la souche étudiée (Michen & Graule, 2010).
- Le second facteur influençant l'immobilisation virale est celui de la composition physico-chimique du sol. Il est aujourd'hui établi que les oxydes métalliques présents dans les sols ; sous forme de cation multivalents ; sont en mesure de retenir très efficacement les virus (Murray & Laband, 1979; Park & Kim, 2015). Les matières organiques du sol sont quant à elles en mesure d'influencer le transport des virus sous forme libre dans le sol, notamment en le favorisant lorsqu'elles sont sous forme soluble (Cao *et al.*, 2010) ou en le freinant lorsqu'elles sont sous forme solide (Schijven & Hassanizadeh, 2000).
- Le dernier facteur à considérer est celui de la solution du sol. En effet la nature des composés chimiques et leurs concentrations respectives ont un impact majeur sur le certaines propriétés de la solution comme son pH (Goyal & Gerba, 1979; LaBelle & Gerba, 1979; Syngouna & Chrysikopoulos, 2010; Taylor *et al.*, 1981), sa salinité (Cao *et al.*, 2010; da Silva *et al.*, 2011; Schaldach *et al.*, 2006) ainsi que la nature des composés minéraux et organiques (Cao *et al.*, 2010; Cheng *et al.*, 2007; da Silva *et al.*, 2011).

La taille réduite des virus entériques, de l'ordre de la dizaine de nanomètres en moyenne (Dowd *et al.*, 1998), rend leur immobilisation majoritairement dépendante d'interactions électrostatiques plutôt que stériques (da Silva *et al.*, 2011; Dowd *et al.*, 1998). L'adsorption des virus sur les agrégats de sol est dès lors médiée par la conjugaison de plusieurs forces telles que les forces de Van der Walls, les interactions diélectriques et hydrophobiques et les forces de Born (Armanious *et al.*, 2016; Dika *et al.*, 2013; Park & Kim, 2015; Van Voorthuizen *et al.*, 2001; Zhang *et al.*, 2014). Alors que les forces de Van der Walls sont toujours attractives, les effets d'attraction et de répulsion des interactions électrostatiques dépendent des charges électriques respectives du virion et de la particule de

sol, elles-mêmes variant selon le point isoélectrique de ces derniers ainsi que le pH et la force ionique de la solution du sol.(Cao *et al.*, 2010; Chattopadhyay & Puls, 2000; Goyal & Gerba, 1979). Comme expliqué plus tôt certains oxydes métalliques présentent des points isoélectriques très élevés, leur permettant de conserver une charge positive sur une gamme de pH étendue, ce qui leur permet de accroître l'adsorption des virions (Syngouna & Chrysikopoulos, 2010), en particulier en présence de cations divalents (Fe^{2+}, Ca^{2+}, Mg^{2+}) et d'une salinité importante (Cao *et al.*, 2010; da Silva *et al.*, 2011; Wong *et al.*, 2012b). Les forces de tension importantes qui s'appliquent dans ce cas peuvent s'avérer suffisantes pour dénaturer la capside protéique et inactiver le virus (Warnes *et al.*, 2015; Zhuang & Jin, 2008). Les interactions hydrophobes, essentiellement liées aux interactions acide-base de Lewis (van Oss, 1993) peuvent très fortement influencer l'interaction résultante (Armanious *et al.*, 2016; Chrysikopoulos & Syngouna, 2012; Dika *et al.*, 2013, 2015). Elles sont très dépendantes du virus (Dika *et al.*, 2013, 2015) et ont une tendance à s'accroître avec la température (Syngouna & Chrysikopoulos, 2010).

Plusieurs théories, telles que celle de la filtration colloïdale (Hunt & Johnson, 2017; Yao *et al.*, 1971) ont uniquement considéré l'immobilisation virale sous sa forme irréversible (Praetorius *et al.*, 2014; Sen, 2011). Aujourd'hui la plupart des théories tiennent compte de la réversibilité de l'adsorption virale à travers la description de sites distincts d'adsorption (Cao *et al.*, 2010), en simulant les cinétiques d'inactivation des virus adsorbés de façon réversible (Tufenkji & Elimelech, 2004) ou à travers la théorie DLVO (Derjaguin-Landau-Verwey-Overbeek) étendue (Hermansson, 1999; Van Oss, 2006). Cette dernière théorie relie la réversibilité de l'adsorption à la distance séparant un soluté (le virus dans notre cas) d'une surface (celle d'un agrégat de sol ou d'un colloïde dans notre cas) (voir **Figure I.6**). Lorsque que cette distance, de l'ordre de quelques nanomètres, est suffisamment faible, le potentiel d'énergie résultant de la somme des forces prises en compte engendre un minimum primaire d'énergie qui attire de façon irréversible la particule virale vers la surface (Praetorius *et al.*, 2014). Dans le cas où la distance virus-surface ne pourrait se réduire qu'à quelques dizaines de nanomètres, la théorie DLVO étendue introduit un minimum secondaire d'énergie, capable de provoquer l'attraction réversible du virus (Cao *et al.*, 2010; Sadeghi *et al.*, 2013; Sasidharan *et al.*, 2017; Tufenkji & Elimelech, 2004). Cette attraction n'existe bien sûr que dans les cas où ce minimum existe (Sadeghi *et al.*, 2013) et que le milieu ne subit pas de changement géochimique important comme une brusque variation de pH ou de salinité (Cao *et al.*, 2010; Tufenkji & Elimelech, 2004).

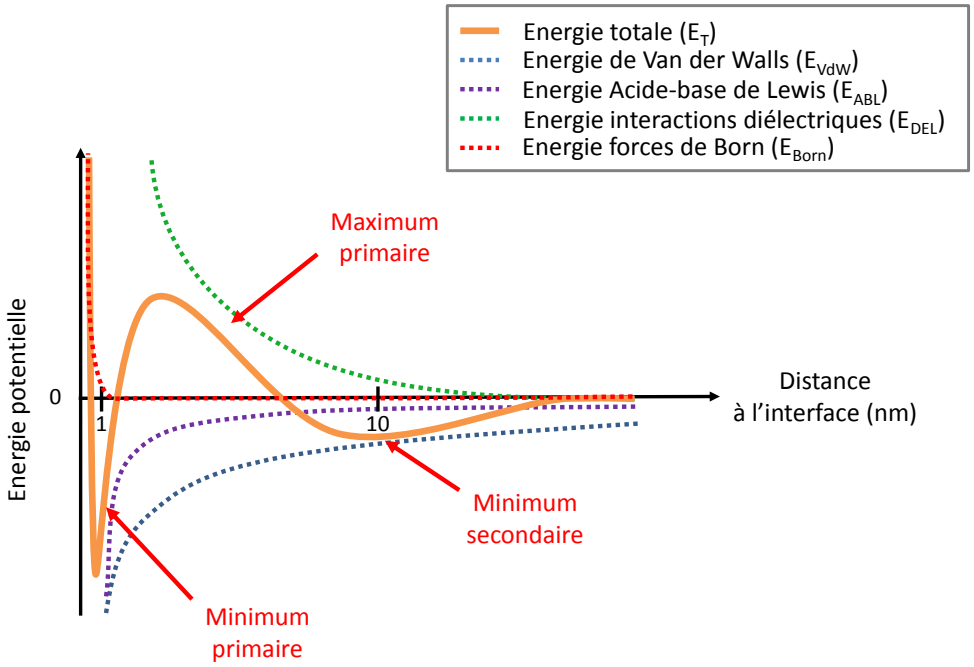


Figure I.6: Forces et interactions moléculaires entrant en jeu dans le cadre de la théorie DLVO étendue. Une énergie potentielle totale, issue de la somme des forces en présence (courbe orange), négative engendre une attraction contrairement à une énergie positive

Dans le cadre des théories le permettant, la réversibilité de l'adsorption peut être décrite à travers différents mécanismes : de façon cinétique *via* des coefficients d'attachement et de détachement (Cao *et al.*, 2010; Dowd *et al.*, 1998; Flynn *et al.*, 2004; Grant *et al.*, 1993; Mayotte *et al.*, 2017; Sadeghi *et al.*, 2013; Sasidharan *et al.*, 2017; Schijven & Šimůnek, 2002; Tim & Mostaghimi, 1991) ; par un coefficient K_D d'équilibre entre virus adsorbés et libres (Anders & Chrysikopoulos, 2009; Cheng *et al.*, 2007; Dowd *et al.*, 1998; Powelson & Gerba, 1994; Sim & Chrysikopoulos, 1999; Syngouna & Chrysikopoulos, 2010; Tim & Mostaghimi, 1991; Torkzaban *et al.*, 2006; Zhuang & Jin, 2008) ; *via* des isothermes de Freundlich (Burge & Enkiri, 1978; Chrysikopoulos & Aravantinou, 2014; Chrysikopoulos & Syngouna, 2012; Gantzer *et al.*, 2001; LaBelle & Gerba, 1979; Powelson & Gerba, 1994; Taylor *et al.*, 1981; Thompson *et al.*, 1998; Zhao *et al.*, 2008) ou de Langmuìr (Grant *et al.*, 1993; Vilker & Burge, 1980).

La réversibilité de l'adsorption virale en fonction du contexte « virus – sol – solution – climat » a été assez peu explorée, notamment dans le cas de sols désaturés, qui peuvent engendrer des forces de tension importantes aux interfaces « air-solide-eau » (Chu *et al.*, 2001; Thompson *et al.*, 1998), lors de la resaturation (Lance *et al.*, 1976) ou d'une modification des conditions géochimiques du sol après l'adsorption des particules virales. Par ailleurs il existe aussi un manque de connaissances concernant les mouvements de particules virales dans des sols « vrais », c'est-à-dire non-remaniés et dont la complexité reflète mieux la réalité.

À cause des nombreuses interactions existantes entre les virions et les particules de sol, ainsi de l'influence de la biologie du sol, la survie des virus sera fortement dépendante du temps passé dans le sol (Hunt & Johnson, 2017) et de sa chimie (Bradley *et al.*, 2011). Ainsi le passage dans le sol d'une solution contaminée par des virus entériques se verra subir un abattement associé aux adsorptions et inactivations subies lors du transport, ce phénomène étant par ailleurs recherché dans le cadre d'une épuration d'eaux usées par passage dans un lit de sable (Aronino *et al.*, 2009; Mayotte *et al.*, 2017; Schaub & Sorber, 1977). Selon les sols cet abattement peut être de 0.001 jour^{-1} jusque 864 jour^{-1} (LaBelle & Gerba, 1979), hors températures extrêmes qui peuvent empêcher tout abattement pendant le temps de l'expérience (Sim & Chrysikopoulos, 1999). L'abattement viral sera bien entendu aussi médié par la composition géochimique du sol et notamment sa teneur en argiles (**Figure I.7**) ou en oxydes métalliques (Tesson & Renault, 2017).

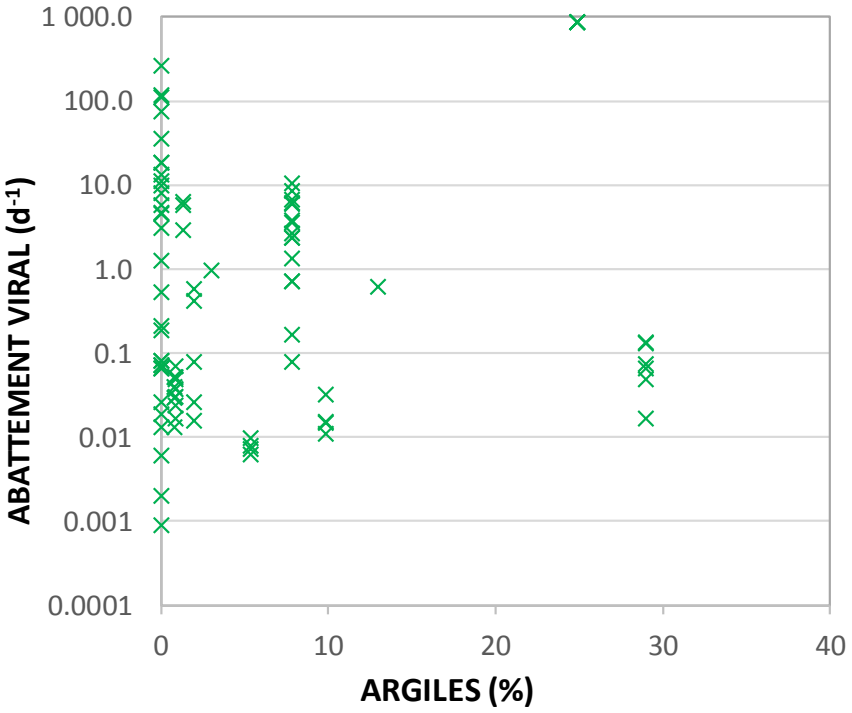


Figure I.7: Abattements viraux dans les sols en fonction des teneurs en argiles telles que rapportés dans la littérature (Tesson & Renault, 2017).

Chapitre II

Proposition et évaluation d'un modèle de production de virus entériques pathogènes de l'Homme et de leur devenir en station d'épuration, en rivières et en nappe d'accompagnement pour simuler les impacts de différents modes de gestion des eaux usées

Ce chapitre a donné lieu à la soumission au journal *Environmental Science & Technology* d'un article intitulé *"Vomiting symptom of acute-gastroenteritis estimated from epidemiologic data can help to assess river contamination by human pathogen enteric viruses"*, cosigné par Tesson V., Belliot G., Estienney M., Wurtzer S., Renault P..

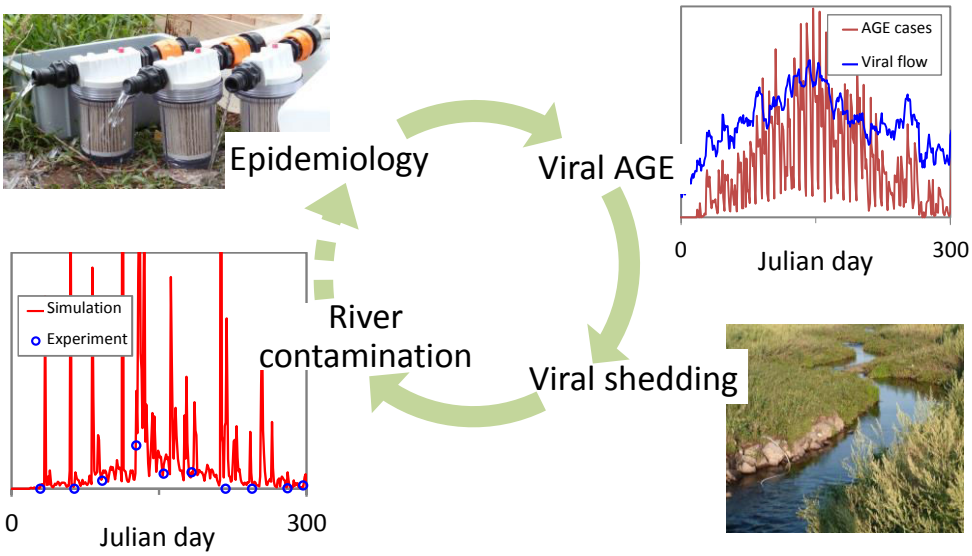
La contamination des compartiments d'eaux douces (rivières, aquifères …) par des virus entériques pathogènes de l'Homme provenant des rejets d'eaux usées est bien établie. Nos objectifs étaient de caractériser tout au long de l'année les contaminations virales des eaux usées et des eaux douces près de Clermont-Ferrand (France), et de vérifier si elles pouvaient être modélisées.

Les norovirus, rotavirus, entérovirus, adénovirus et virus de l'hépatite A ont été quantifiés par méthodes moléculaires après concentration de l'eau. Les écoulements d'eau ont été obtenus à partir de la banque de données Hydro et de la station d'épuration des eaux usées. Les cas déclarés de gastro-entérites aigües et les prescriptions médicales ont été fournis par *Santé Publique France*. Nous avons estimé le nombre quotidien total de gastro-entérites aiguës d'étiologie virale, et modélisé l'excrétion virale et le devenir des virus en station d'épuration et dans les rivières. Les concentrations simulées de virus ont été comparées à la somme pondérée des concentrations mesurées. Les critères de vomissement ont permis de simuler les variations saisonnières des gastro-entérites aiguës d'étiologie virale. À l'exception du virus de l'hépatite A, tous les virus ont généralement été détectés dans les eaux usées et les rivières, avec des concentrations atteignant 10^{+8} $GC.L^{-1}$ pour les adénovirus dans l'Artière. Il a été possible de simuler la charge virale des eaux usées brutes et de l'Artière en aval du point de rejet des eaux usées traitées ; les coefficients de pondération estimés ont montré l'impact élevé des norovirus GII. De telles simulations permettent de comparer des scénarios de traitements, de rejets et/ou de réutilisations; acquises en temps réel, elles permettraient de réguler la désinfection UV supplémentaire.

Les scénarios envisageables sont divers : ils peuvent concerner la proportion de rejet et de recyclage, la possibilité ou non de stockage hivernal d'eau usée traitées dans de grands réservoirs, le(s) type(s) de réutilisation(s) des eaux usées, et la possibilité en option de traitements tertiaires additionnels (rayonnements UV notamment car permettant plus facilement d'adapter le traitement à la contamination supposée de l'eau). Au-delà, l'évaluation quantitative des risques microbiens associés à la présence de virus doit inclure des risques associés à la consommation d'eau de boisson (éventuellement prélevées dans la nappe alluviale d'accompagnement des rivières considérée avec un effet filtre des milieux poreux traversées) puis traitées et à la pratique d'activités aquatiques récréative sur l'Allier ou sur toute étendue d'eau en contact direct avec l'Allier.

Ce modèle pourrait être enrichi à travers le suivi d’autres critères discriminants pour l’étiologie, en particulier les diarrhées dites muqueuses et la fièvre. De la même façon, l’identification des zones à risques en aval de la confluence entre Artière et Allier (captages d’eau potable, activités nautiques ou pêche) augmenterait grandement la pertinence de l’analyse quantitative des risques microbiens.

Graphical abstract:



1. Abstract:

The contamination of water bodies by human enteric viruses coming from wastewater discharges is well established. Our objectives were to characterize throughout the year virus contaminations of wastewaters and freshwaters near Clermont-Ferrand (France), and check whether they could be modelled. Noroviruses, rotaviruses, enteroviruses, adenoviruses and hepatitis A viruses were quantified by molecular methods after water concentration. Water flows were obtained from the Hydro databank and the treatment plant. Declared acute gastroenteritis cases and medical prescriptions were provided by Santé Publique France. We estimated the total number of daily viral acute gastroenteritis, and modelled virus shedding and fate in treatment plant and rivers. Simulated virus concentrations were compared to the weighted sum of measured concentrations. Vomiting criteria allowed simulating seasonal variations in viral acute gastroenteritis. For all but hepatitis A virus, all viruses were generally detected in wastewaters and rivers, with adenovirus concentrations reaching 10^{+8} GC.L^{-1} in the Artière River. It was possible to simulate virus load of raw wastewater and Artière River; estimated weighting coefficients showed the high impact of noroviruses GII. Such simulations could be useful for the comparison of scenarios of treatments, discharge and / or reuse; when acquired in real time, they would make it possible to regulate additional UV disinfection.

Key words: human enteric viruses, river contamination, acute gastroenteritis, bioindication, risk assessment

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2. Introduction:

The contamination of freshwater bodies by human pathogen enteric viruses is now well established. Rivers are nearly systematically contaminated: the concentrations in viral genome copy (GC) could reach 10^{+4}, 10^{+4} and 10^{+6} $GC.L^{-1}$ for noroviruses, rotaviruses and adenoviruses, respectively (D'Ugo *et al.*, 2016; Prevost *et al.*, 2015b; Sinclair *et al.*, 2009; Vergara *et al.*, 2016), while presenting seasonal variations in quantity (Iaconelli *et al.*, 2016; Vieira *et al.*, 2016) and in diversity (Ogorzaly *et al.*, 2015). Similarly, contaminations of underground and source waters have often been noted (Lodder *et al.*, 2010; Murphy *et al.*, 2017), where viruses can remain infectious for a long time (e.g. up to 120 days for human adenovirus while its DNA remained detectable for up to 400 days (Ogorzaly *et al.*, 2010), and up to 61 days for noroviruses while its RNA remained detectable for up to 1266 days (Seitz *et al.*, 2011)). While viral concentrations in groundwater have long been assumed to be negligible, such observations led to regulatory changes in the U.S. in 2006 (Reynolds *et al.*, 2008). In addition, some studies have demonstrated that human enteric viruses in rivers come directly from wastewater discharges despite treatments (Prevost *et al.*, 2015b), virus quantities and diversities in wastewaters varying with the seasons (Brinkman et al., 2017 ; Prevost et al., 2015). Models combining the shedding of viruses and their fate in sewers, treatment plants and rivers could help assess the sanitary, environmental and economic impacts of additional treatments or the replacement of the discharge of treated wastewater through reuse in strongly water-dependant activities (e.g. agriculture).

In 2010, about 40% of the World population (2.8 billion people) had access to improved sanitation, i.e. connections to sewerage systems with the subsequent treatment of wastewaters prior to their discharge in the environment (Baum *et al.*, 2013); such proportion was higher in industrialized countries: e.g. 74, 95 and 95% in France, UK and Germany, respectively (Baum *et al.*, 2013). It has quickly increased in several countries (e.g. from 1.4 to 39.7% in Cyprus, and from 16 to 78% in Greece between 1990 and 2010 (Baum *et al.*, 2013)), and the not concerned population in rural or suburban areas may have access to individual household sanitation (Berendes *et al.*, 2017): e.g. about 17% of the French population in 2008 (French Ministry of Ecological and Solidarity Transition, 2011). Virus concentrations in sewers may be affected by leakages (Gotkowitz *et al.*, 2016; Roehrdanz *et al.*, 2017), by dilution with stormwaters in combined sewerage systems, and often by reducing conditions and/or iron oxides resulting from the alteration of concretes (Hvitved-Jacobsen *et al.*, 2013) when the residence time in sewer is high (the median residence time is about 3.3 h in the U.S. (Kapo *et al.*, 2017), and Nielsen et al. (Nielsen *et al.*, 1992) considered that wastewater flow velocities vary typically between 0.5 and 1 $m.s^{-1}$). By contrast in rivers, we may neglect virus removal over a few hours corresponding to distances traveled from a few km to a few tens of km (Miller *et al.*, 1994). During rainy periods and for combined sewerage systems that collect stormwaters, the bypass leads to discharges of raw wastewaters into the environment, whereas they are treated in wastewater treatment plants in other situations.

Several studies have dealt with virus removal in wastewater treatment plants leading to meta-analyses (Pouillot *et al.*, 2015; Sano *et al.*, 2016) or reviews (Campos *et al.*, 2016; Verbyla & Mihelcic, 2015), and a model has been compared to experimental data (Haun *et al.*, 2014). Unfortunately, meta-analyses ignore several parameters that affect virus removal, including activated sludge tank aeration, pore sizes of membranes in membrane bioreactor, and chemicals for microbial sludge flocculation (Haun *et al.*, 2014) that may affect virus co-precipitation. Classical pre-treatments and activated sludge treatments that are the most common in wastewater treatment plant allow human enteric virus removal of generally between less than 1 $\log_{10}$ (La Rosa *et al.*, 2012) and 3-4 $\log_{10}$ (Campos *et al.*, 2016), but virus removal greatly depends on viruses themselves (da Silva *et al.*, 2007). When existing, additional physical treatments (filtrations, UV exposure) and/or chemical treatments (chlorine, ozone, hydrogen peroxide, peracetic acid) lead to additional virus removal (Verbyla & Mihelcic, 2015). Lagooning that is preferred to activated sludge treatment in some villages can also be used as an additional treatment contributing to virus removal (Verbyla & Mihelcic, 2015) in greater cities.

Human pathogen enteric viruses multiply in humans exclusively and are excreted with their stools, except for some viruses that also infect animals, such as hepatitis E viruses (Koopmans & Duizer, 2004) and severe acute respiratory syndrome viruses (Gundy *et al.*, 2009). Among them, human noroviruses are one of the leading causes of acute gastroenteritis (Kirk *et al.*, 2015; Yeargin *et al.*, 2016) (36% of gastroenteritis in the World, 67% in Europe), that both the *US Environmental Protection Agency* and the *American Water Works Association* (Hoffman *et al.*, 2009) recommended to monitor in drinking water, and that the *Codex Alimentarius Commission* recognized as one of the most important microbial risks (FAO, 2012). Teunis et al.(Teunis *et al.*, 2015) showed that norovirus GII.4 concentrations could reach about 10^{+10} GC.g^{-1} faeces 1-2 days after infection and could still exceed 10^{+2} GC.g^{-1} faeces after between 55-75 days, long after the end of the symptoms; they also noted that virus shedding was similar for symptomatic and asymptomatic carriers (about 70 and 30% of the investigated population (Glass *et al.*, 2009; Teunis *et al.*, 2015), respectively). Other studies carried out on norovirus shedding yielded similar results (Atmar *et al.*, 2008; Furuya *et al.*, 2011; Lee *et al.*, 2007; Milbrath *et al.*, 2013; Newman *et al.*, 2016b; Sabrià *et al.*, 2016). Linking public health data to the etiology of acute gastroenteritis faces two types of difficulties: (i) the use of statistical data dealing only with people consulting a physician (Van Cauteren *et al.*, 2012), and (ii) the absence of symptoms specific to the etiology (Arena *et al.*, 2014). However, some studies make it possible to estimate the whole gastroenteritis cases from the cases with a prescription, e.g. in France (Van Cauteren *et al.*, 2012). Furthermore, some symptoms that vary statistically over populations with the etiology may *a priori* allow to weight the etiologies of acute gastroenteritis in populations of significant size: especially in France during the consecutive winters 2010-2011 and 2011-2012, vomiting concerned 66 and 52% of the patients consulting a general practitioner with at least one virus and no virus detected in stools, respectively (Arena *et al.*, 2014). While vomiting may be identified from prescriptions, fever more frequent in viral infection (46% vs 33%) and mucous diarrhea less

frequent in viral infection (11% vs 17.4%) (Arena *et al.*, 2014) unfortunately cannot be identified from these prescriptions. One last indication that may be useful is the seasonality of viral gastroenteritis with a winter maximum: in Poland, they account for 87% and 55% of viral or bacterial gastroenteritis in February and July, respectively (Baumann-Popczyk *et al.*, 2012). Only enteroviruses (including coxsackie viruses and echoviruses) have a summer maximum (Dowell, 2001; Moore *et al.*, 1988). The possibility of using public health real-time data to estimate viral shedding would make it possible to circumvent the problems of cost, delay and feasibility of some detection and quantification methods (e.g. those based on the propagation of noroviruses on target cell cultures (Ettayebi *et al.*, 2016), even if other methods try to circumvent the problem (Karim *et al.*, 2015; Prevost *et al.*, 2016)), and the lack of relevant bioindicator of viral contaminations despite numerous studies devoted to this topic (Wong *et al.*, 2012a).

The objectives of this work were (i) to characterize throughout the year viral contaminations of wastewaters and of surface and underground waters potentially affected by these wastewaters, and (ii) to check whether such variations could be simulated by a model taking into account human viral shedding indirectly estimated from epidemiological data, viral removal in *Clermont-Auvergne-Métropole* WWTP and dilutions in rivers.

3. Materials and Methods

a. Experimental context

The work was carried out on a small geographical area around the French Clermont-Ferrand city (45.77 N, 3.09 E), which consists of 3 distinct subunits (**Figure II.1**): the collection basin of wastewaters conveyed to the *'Trois Rivières'* wastewater treatment plant, the Artière watershed and a small part of the Allier watershed that affect the flows and contaminations of these rivers (by surface runoff and by exchanges between these two rivers and their alluvial aquifers), and the area irrigated by treated wastewater and managed by the *'ASA Limagne Noire'* association bringing together 51 farmers which is the most important French site for wastewater reuse in crop irrigation (1500 ha equipped with a pumping station, a 60-km water distribution network and 150 connections). The wastewater collection basin includes the 19 towns and villages (i.e. 17 and the industrial zone of an eighteenth city among the 21 cities of the *Clermont-Auvergne-Métropole* and a nineteenth city out of this agglomeration,) corresponding to a total 237 700 inhabitants (according to the 2014 French official census), whose wastewaters are collected by the *'Trois Rivières'* wastewater treatment plant.

The new *'Trois Rivières'* wastewater treatment plant was designed and build by Degrémont in 2003-2004 on the site of the former wastewater treatment plant to treat 425 000 population-equivalent (PE), although it currently treats only 200 to 320 000 PE (i.e. about 11 million $m^3.year^{-1}$). The treatment train operated by Veolia includes a pre-treatment

(screening followed by sand and oil removals), and a conventional biological low load activated sludge process with enhanced nitrification/denitrification and phosphate elimination. The use of iron chlorosulfate to precipitate phosphates can favour the partial immobilization of viruses. Treated wastewaters are discharged into the Artière river, but since 1996, some of them have undergone tertiary treatment before being reused in agricultural irrigation (mainly for corn seeds and sugar beets). To this end, eight lagoons owned by the industry *'la Sucrerie de Bourdon'* to treat from October to April its seasonal industrial effluents (cleaning of sugar beets) before their use for irrigation are operated by the farmer association from May to September for additional wastewater tertiary treatment. Only 700 ha are irrigated each year with wastewaters to ensure at least two weeks of lagooning. The average annual reused water volume is 1 106 000 $m^3.year^{-1}$ (i.e. about 1600 $m^3.ha^{-1}.year^{-1}$). The reused water flow rate is 1000 to 1400 $m^3.hour^{-1}$ (18 000 to 25 000 $m^3.day^{-1}$ during peak water demand) representing 30 to 40% of the wastewater treatment plant effluent flow rate (COTITA Centre-Est *et al.*, 2011). Upstream the discharge by the *'Trois Rivières'* treatment plant, the Artière River received treated wastewater from a small treatment plant (120 PE); the Allier River and its tributaries upstream from the confluence with the Artière River also receive treated wastewaters sewage from many small cities.

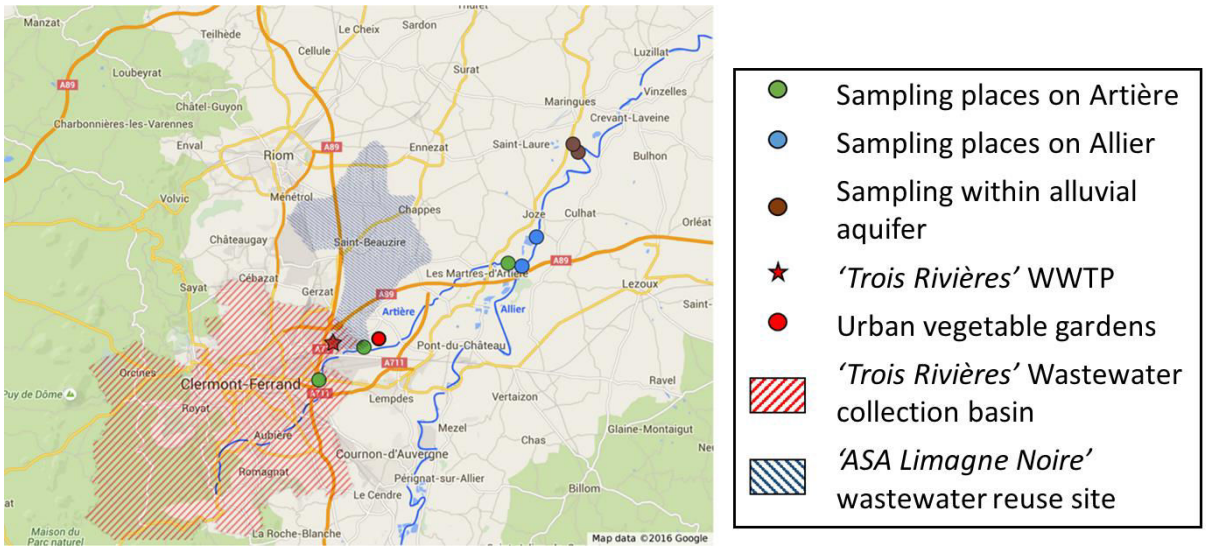

Figure II.1: Map of the study area delineating the sewage collection basin, the area irrigated by wastewater and the sampling points. *(Punctual sampling of vegetables and borehole water were performed in some vegetable gardens near the Artière river, boreholes being located at about 50 to 300 m of the Artière bank).*

The climate is particularly affected by the *'Chaine des Puy'* mountains at the west of the area; it is semi-continental (i.e. a mixture between oceanic and continental climate). Mean annual rainfalls greatly vary from West to East on a few km scale with a maximum of more than 1000 mm of water in the *'Chaine des Puy'* mountains near which the source of the

Artière is located, and minimum of less than 600 mm in the *'ASA Limagne Noire'* area (Hydratec, 2015). During the monitoring period (i.e. from June 2015 to August 2016) at the Aulnat's climatic station within Clermont-Ferrand airport, atmospheric average daily temperatures varied between 0.3 and 27.9°C (annual mean from September 2015 to August 2016 of 12.3°C), and the cumulated rainfalls reached 685 mm, with an annual cumulated rainfall from September 2015 to August 2016 of 564 mm precipitations. During the same period, the flow of Artière upstream of treated wastewater discharge varied between 5184 and 140 832 $m^3.day^{-1}$ and was generally more than doubled by treated water discharge (that varied between 12 125 and 266 054 $m^3.day^{-1}$), i.e. the mean downstream Artière flow – to – upstream Artière flow ratio being equal to 5.2. During the same annual period, the wastewater treatment plant by-pass was operated 60 days with maximum discharge flow of 10 500 $m^3.day^{-1}$. By contrast, the flow of the Allier River upstream of the confluence with the Artière varied between 1 071 360-19 267 200 $m^3.day^{-1}$ (and 1 121 288 and 19 441 286 $m^3.day^{-1}$ at Limons station), and was only marginally increased by the Artière River confluence (between 0.5 and 11% of the Allier flow downstream the confluence).

b. Experimental design and protocols

The experimental monitoring were carried out between 1st June 2015 and 31 August 2016, with sampling once a month and once every two weeks outside and during the period of irrigation by treated wastewater, respectively. Wastewaters samplings concerned (**Figure II.1**) raw wastewater just after screening (i.e. before sand and oil removals), treated wastewater at the outlet of the wastewater treatment plant, and treated wastewater at the outlet of the last (eighth) lagoon during the irrigation period. Conventional surface waters were sampled in the Artière River upstream and downstream of the treatment plant discharge and just before the confluence with the Allier River, and in the Allier River upstream and downstream from this confluence. Underground conventional waters were sampled within the Allier alluvial aquifer 7.5 km downstream of the confluence with the Artière River in wells used for agricultural irrigation at 80 and 400 m from the Allier bank. Additional measurements performed simultaneously concerned the temperature and the pH of the waters.

Environmental concentrations in noroviruses (genogroups I and II), rotaviruses, enteroviruses, adenoviruses and hepatitis A viruses were monitored by using a procedure of water sampling, virus concentration and purification nearly identical to the procedure of Prevost et al. (2015b) that derived from the method 1615 of the US-Environmental Protection Agency (Cashdollar *et al.*, 2013; Gibson *et al.*, 2012). For each sampling of conventional surface or groundwater, 100 L of water were pumped at a rate of 2 L. $minute^{-1}$ with a SMART L40 3-roller peristaltic pump (Verderflex®, Castleford, U.K.) using Verderprene® tubes 19 × 4.8 mm, and filtered *in situ* on Argonide® filter cartridges NanoCeram® VS2.5-5 (Argonide Corporation, Sanford, FL, U.S.) having a surface area of 12.9 dm^2 within H2.5-5C Clear-Housing; NanoCeram® are electropositive filters with alumina fiber of 2 nm as active

phase. After having been emptied of water, the filter cartridges within their Clear-Housing were returned under cold conditions to the laboratory. For each wastewater sampling (at the inlet and outlet of the wastewater treatment plant, and within lagoons), 1 L of water was stored in borosilicate bottles and returned in the Lab under cold conditions; it was then filtered on 90 mm non-laminated NanoCeram disks at about 100 mL.minute^{-1} water flow to ensure approximately the same flux of water with regard to filter surface, by using glass filter holder kit from Millipore.

Viruses were eluted from the filter using an elution buffer (0.05 M glycine, 1% w/v Bacto Beef Extract, 0.1% Na triphosphate, 0.1% Tween, 0.1% Silicon Emulsion Anto-foam) adjusted at pH=9.5 just before its use. 450 and 50 mL of this buffer were poured into each of the Clear-Housing containing their filter cartridge and into 50 mL Falcon tubes already containing the filter discs cut into small pieces, respectively. Clear-Housing and Falcon tubes were immersed during 1 h in an ultrasonic bath XUB 18 (Grant Instruments Ltd., Royston, UK) previously filled with fresh water and ice cubes to ensure a water temperature always below 10°C, at 16 W.L^{-1}. The buffer solutions with eluted viruses were then transferred in 500 mL glass bottles and 50 mL Falcon tubes, and their pH was adjusted to 3.5 with concentrated HCl. They were gently stirred during 1 h at the laboratory temperature using a Reax 2 rotary shaker at minimum speed (Heidolph Instruments GmbH & Co, Walpersdorfer, Germany). The solutions were then centrifuged during 2 h at 4500 g and 4°C, using a centrifuge 6K15 (Sigma, US). After centrifugation, the pellet was resuspended in 8 mL phosphate buffer (0.15 M Na_2HPO_4 at pH=9.0) and centrifuged during 15 min at 4500 *g* and 4°C to clarify the viral concentrated suspension. The supernatant was filtered on a low-binding Millex HV-HPF 0.45 µm, 25 mm in diameter. The viral suspensions were concentrated one last time and their beef extract that inhibit PCR partly removed by ultracentrifugation during 2 h at 143 000 g and 4°C on sucrose cushion (1.5 mL at 40% sucrose) on a L8-70M Ultracentrifuge (Beckman-Coulter Inc., US) using a SW41Ti rotor. The supernatant was then eliminated with at least 0.5 mL of the sucrose cushion to minimize the amount of remaining beef extract. The pellet was resuspended in the 0.6 ml remaining sucrose cushion.

Detection and quantification of viral genome copies of noroviruses (genogroups I and II), rotaviruses, enteroviruses, adenoviruses and hepatitis A viruses were performed according to the procedure of Prevost et al. (2015b), except for an additional step of PCR inhibitors removal using One-Step PCR inhibitor removal kit (ref. D6030, Zymo research, USA) according to manufacturer instructions. Primers and probes employed for molecular detection were designed as previously described (Prevost *et al.*, 2015b), excepted for Norovirus GI probe which was corrected as following: FAM-CGA{T}CYCC{T}GTC{C}ACA-BHQ1. Detection thresholds were about $10^{2.1}$, $10^{0.4}$, $10^{2.2}$, $10^{0.5}$, $10^{2.6}$ and $10^{1.6}$ GC.L^{-1} for noroviruses GI, noroviruses GII, rotaviruses, enteroviruses, adenoviruses and hepatitis A viruses, respectively when 1 L of water was sampled; those thresholds were *a priori* inversely proportional to the volume of water.

Additional measurements of the pH and temperature of water were performed *in situ*, and other aliquots of water were sampled for additional analyses: 500 mL for *Escherichia coli* quantification at the *'Laboratoire Départemental d'Analyse'* of Vaucluse in Avignon (France), 180 mL for total organic C and N measurements, 200 mL for anions and cations measurements, 40 mL for NH_4^+ measurements.

c. Water flow data

Additional data concerning the flow of rivers (Crouel on Artière River upstream discharge of wastewater (45.771548 N, 3.1424498 E), Vic-le-Comte on Allie River r upstream the Artière River confluence (45.661528, 3.2020719) and Limons on Allier River downstream the Artière River confluence (45. 968896, 3.448480)) were obtained from the Hydro databank of the French *'Ministère de l'Ecologie, du Développement Durable et de l'Energie'* (Ministère de l'Ecologie, du Développement Durable et de l'Energie, 2015). Data relating to the wastewater treatment were provided by the wastewater treatment plant with the agreement of *Clermont-Auvergne-Métropole*. We assumed that the Artière River flow both downstream wastewater discharge and near the Allier River confluence were equal to the Artière River flow upstream the discharge decreased by water withdrawal for filling the lagoons. By doing this, we neglected other contributions to River flows, including small stream contribution (mainly Le Bec stream), surface runoff during rainy periods and exchanges between rivers and alluvial aquifers.

d. Epidemiologic data

Acute gastroenteritis cases and adapted drug data estimated through the reimbursement of medical expenses by the French Social Security and reported during the viral monitoring period and the previous 6 years were kindly provided by *Santé Publique France*, the French national public health agency. For each person with gastroenteritis who consulted a doctor, available information includes the age group (either 1-4, 5-65 or >65 years old), the residence place, and the medical prescriptions (antiemetic, antidiarrheal, both antiemetic and antidiarrheal, oral rehydration solutions (ORS) for <1 year or 1-15 years children, and others). Prescriptions entitled "Others" include antispasmodics and intestinal adsorbents. Note that antiemetics are very rarely prescribed, or even contraindicated, for children; for this reason, the proportion of vomiting people among those consulting a physician were estimated without taking into account people having ORS and "Others" prescriptions.

Epidemiologic and drug data were analysed during the period of virus monitoring. Total declared acute gastroenteritis cases with the optional distinction of prescriptions were quantified for each of the three age groups and related to the size of these groups according to the French official census of 2014 (i.e. 12 100, 184 600 and 41 000 inhabitants of 1-4, 5-65

and >65 years old, respectively, for the 19 towns included in the wastewater collection basin arriving at the *'Trois Rivières'* wastewater treatment plant). After preliminary tests (results not shown), their temporal variations were described using sliding averages over 7 days to avoid insignificant fluctuations (associated with the randomness of infections) while avoiding erasing true temporal trends (**Figure II.2**).

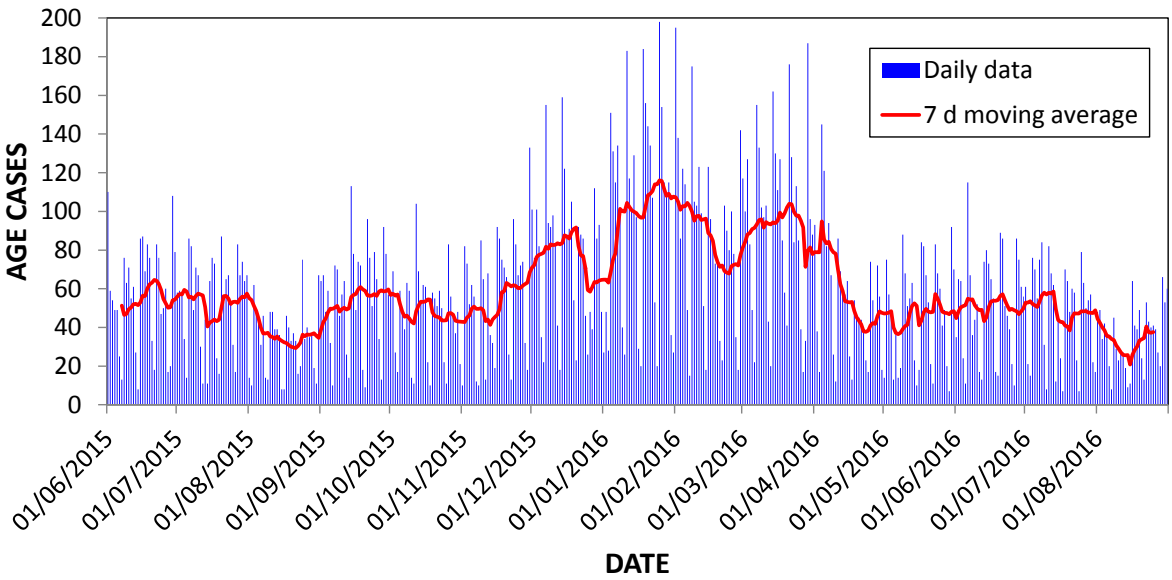

Figure II.2 : Comparison between actual daily data concerning all acute gastroenteritis (AGE) and the trends reflected by 7 d sliding averages.

We investigated correlations in acute gastroenteritis cases between age groups, prescriptions, and physician's office places without sliding averages. Periods in which acute gastroenteritis and/or prescribing involved all three age groups or only one of these age groups were identified, as differences in epidemiology between age groups could result from either immunization or contamination route.

We assumed that the daily proportion of viral aetiology for people suffering from acute gastroenteritis $p_{AGV}(v,d)$ during the Julian day d could be estimated from the daily proportion of sick people with vomiting symptoms $p_{AGE}(vom.,d)$, as vomiting symptoms are more frequent for infections by viruses than for infections by bacteria (Arena *et al.*, 2014). Considering the probabilities of vomiting symptoms $p_{AGE|v}(vom.)$ and $p_{AGE|b}(vom.)$ for sick people infected by viruses and bacteria, respectively, and neglecting other possible aetiologies of acute gastroenteritis, the proportions of acute gastroenteritis caused by viral infections can be estimated with the following equation for $p_{AGE|b}(vom.) \leq p_{AGE}(vom,d) \leq p_{AGE|v}(vom.)$:

$$p_{AGE}(v,d) = \frac{p_{AGE}(vom.,d) - p_{AGE|b}(vom.)}{p_{AGE|v}(vom.) - p_{AGE|b}(vom.)} \quad (1)$$

$p_{AGE}(v,d)$ was set at 0 for $p_{AGE}(vom,d) \leq p_{AGE|b}(vom.)$ and to 1 for $p_{AGE|v}(vom.) \leq p_{AGE}(vom,d)$. $p_{AGE|b}(vom.)$ and $p_{AGE|v}(vom.)$ were set at 0.52 and 0.66, respectively, in agreement with Arena et al.(2014). Since we did not have direct access to the daily proportion $p_{AGE}(vom.,d)$, but only to the corresponding daily proportion of vomiting symptoms among people consulting a physician $p_{AGE|consult}(vom.,d)$, $p_{AGE}(vom.,d)$ was estimated from $p_{AGE|consult}(vom.,d)$ and the probabilities $p_{AGE|vom.}(consult.)$ and $p_{AGE|\overline{vom.}}(consult.)$ to consult a physician when suffering from vomiting symptoms or not, respectively:

$$p_{AGE}(vom.,d) = \frac{-p_{AGE|consult.}(vom.,d) \times p_{AGE|\overline{vom.}}(consult.)}{p_{AGE|consult.}(vom.,d) \times [p_{AGE|vom.}(consult.) - p_{AGE|\overline{vom.}}(consult.)] - p_{AGE|vom.}(consult.)} \quad (2)$$

Two alternative hypotheses were used to estimate $p_{AGE|vom.}(consult.)$ and $p_{AGE|\overline{vom.}}(consult.)$:

- $p_{AGE|vom.}(cons.)$ and $p_{AGE|\overline{vom.}}(cons.)$ were equal, as Van Cauteren et al.(2012) did not include vomiting and nausea symptoms as determinants of consultation in their final multivariate logistic regression. This led assuming that the proportions of sick people presenting vomiting symptoms are identical in the whole set of sick people and in the subset of sick people consulting a physician. $p_{AGE|vom.}(cons.)$ and $p_{AGE|\overline{vom.}}(cons.)$ were then set to 0.334 as proposed by Van Cauteren et al. (2012);
- $p_{AGE|vom.}(consult.)$ and $p_{AGE|\overline{vom.}}(consult.)$ were estimated from the following equations:

$$p_{AGE|vom.}(consult.) = \frac{p_{AGE}(consult.)}{p_{AGE}(vom.)} \times p_{AGE|consult.}(vom.) \quad (3a)$$

$$p_{AGE|\overline{vom.}}(consult.) = \frac{p_{AGE}(consult.)}{p_{AGE}(\overline{vom.})} \times p_{AGE|consult.}(\overline{vom.}) \quad (3b)$$

where $p_{AGE}(consult.)$ and $p_{AGE}(vom.)$ are the probabilities of people suffering from acute gastroenteritis to consult a physician or to vomit, respectively. $p_{AGE}(consult.)$ and $p_{AGE}(vom.)$ were set at 0.334 and 0.629 (Van Cauteren *et al.*, 2012), and $p_{AGE|consult.}(vom.)$ and $p_{AGE|consult.}(\overline{vom.})$ were estimated from *Santé Publique France* data (0.79 and 0.21, respectively). Estimates of $p_{AGE|vom.}(consult.)$ and $p_{AGE|\overline{vom.}}(consult.)$ were then 0.419 and 0.190, respectively.

The total number of daily viral acute gastroenteritis *n(AGE|v)* was estimated from the proportion of viral gastroenteritis $p_{AGE}(v)$, the total number of medical consultations *n (AGE|consult.,d)* and the likelihood of seeing a physician in case of acute gastroenteritis:

$$n(AGE|v,d) = p_{AGE}(v) \times n(AGE|consult.,d) \times \left(\frac{1}{p_{AGE}(consult.)}\right) \quad (4)$$

e. Viral shedding, transport and removal modelling

Virus shedding of symptomatic and asymptomatic carriers was described by the model of norovirus concentrations in stools *C(t)* (GC.g^{-1} stool) proposed by Teunis et al. (2015):

$$C(t) = C_{max} \times fac \times e^{-\alpha \times (t)} \times \left(1 - e^{-(\beta-\alpha)\times(t)}\right) \quad (5)$$

where *t* is the time (day) after the beginning of the acute gastroenteritis, C_{max} is the maximum virus concentration (GC.g^{-1} stool), α (day^{-1}), β (day^{-1}) and *fac* three constants linked with each other as proposed by Teunis et al. (2015) (cf. their supporting information). C_{max}, α, β and *fac* values were set to 10^{+7} GC.g^{-1} stool, 0.53 day^{-1} , 0.48 day^{-1} and –27.2, respectively, in order to fit with the median relationship proposed by Teunis et al. (2015). We assumed that asymptomatic virus carriers excrete 100 g stool.day^{-1}, and that stool excretion increased up to 500 g stool.day^{-1} during the first 3 days of acute gastroenteritis for symptomatic carriers (Petterson *et al.*, 2016). The total daily quantity of virus shedding per person $Q_{p\text{-}sympt.}(v,t)$ (GC.day^{-1}.p^{-1}) from one symptomatic carrier was then estimated from virus concentration within stools and the daily quantity of stool excreted:

$$Q_{p-sympt}(v,t) = C(t) \times m_{stool-sympt.}(t) \quad (6)$$

where $m_{stool\text{-}s}(t)$ is the daily quantity of stool excreted *t* days of acute gastroenteritis. Although the proportion of asymptomatic carriers $p_{AGE}(asympt.)$ and their own virus shedding depend on the virus (Jacobsen & Wiersma, 2010; Mukhopadhya *et al.*, 2013; Pintó *et al.*, 2010), we decided to use data available for norovirus GII, as it explained most of the experimental trends (Rivière *et al.*, 2017) (see *Results and Discussion* section in this paper). $p_{AGE}(asympt.)$ was set to 0.30 (Teunis *et al.*, 2015). The total amount of virus shedding for one symptomatic carrier and the corresponding proportion of asymptomatic carrier $Q_p(v,t)$ (GC.day^{-1}.p^{-1}) was then:

$$Q_p(v,t) = C(t) \times \left(m_{stool-sympt.}(t) + \left(m_{stool-asympt.}(t) \times \left(\frac{p(asympt.)}{1-p(asympt.)}\right)\right)\right) \quad (7)$$

The daily flow of viruses at the inlet of the *"Trois Rivières"* wastewater treatment plant $Q(v,t)$ (GC.day^{-1}) could then be estimated with the following equation:

$$Q(v,d) = \sum_{t=0}^{n_{day}} Q_p(v,t) \times n(AGE|v, d + d_{consult} - d_{sew} - t) \quad (8)$$

where n_{day} is the number of days of virus shedding, $d_{consult}$ the delay between the onset of shedding and physician consultation (day), and d_{sew} is the residence times of wastewater in sewer (day). As $d_{consult}$ average was about 1.5 days (Van Cauteren *et al.*, 2012) and a rough estimation of d_{sew} was about 2 days (assuming that 10% of the 1000 km of 50 cm internal diameter sewer pipes are filled, and a daily wastewater flow of 53 000 m^3.day^{-1}), we neglected the difference $d_{consult} - d_{sew}$. Having no information on the fate of viruses in the sewers, we ignored virus removal within the sewerage system, but we have discussed the validity of this hypothesis given the differences between experiments and simulations. To enable

comparisons between virus concentrations simulated without distinguishing viral species and experimental data for various enteric viruses, we defined equivalent virus concentrations $[v_{eq},d]$ (GC.L^{-1}) from these last measured concentrations:

$$[v_{eq},d] = a \times [NoGI,d] + b \times [NoGII,d] + c \times [RoV,d] + d \times [EnV,d] + e \times [AdV,d] \quad (9)$$

where $[NoGI,d]$, $[NoGII,d]$, $[RoV,d]$, $[EnV,d]$ and $[AdV,d]$ are the concentrations (GC.L^{-1}) in noroviruses GI, noroviruses GII, rotaviruses, enteroviruses and adenoviruses, respectively, and a, b, c, d and e five weighting coefficients that express their supposed relative impact per viral particle on acute gastroenteritis. These coefficients were estimated by fitting equivalent virus concentrations to simulated virus concentrations in raw wastewater, i.e. by minimizing the sum of the squares of the differences between viral concentrations simulated from epidemiological data and equivalent virus concentrations issued from equation (9). The same coefficients were used thereafter to compare measured virus concentrations to simulated virus concentrations in Artière and Allier rivers.

We assumed that virus removal between raw wastewater at the inlet of the wastewater treatment plant and any sampling place downstream in rivers occurred only within the treatment plant. Indeed, the distance between the outlet of the treatment plant and the furthest sampling place on the Allier is approximately 15 km, which may be reached by contaminants leaving the treatment plant in about 4 hours. This time is usually too short for noticeable virus removal within rivers, as already noted on a 80-90 km stretch of the Seine River around Paris (Prevost *et al.*, 2015b). Experimental virus removals within the wastewater treatment plant were estimated for each of the quantified viruses at each sampling date, as the virus inflow – to – virus outflow ratio; the virus outflow was estimated from the increase in the virus flow in the Artière River resulting from treated wastewater discharge rather than from virus concentrations in treated wastewaters. Seasonal variations in virus removal were checked and geometric averages were calculated over the 1st September 2015 – 31st August 2016 period. In addition, as raw and treated wastewater samplings were performed at about 9-10 h UT, we checked whether our experimental protocol induced a bias in the estimates of the virus removal rates, assuming removal rates at each stage of wastewater treatment that insured a cumulated removal near the experimentally estimated one (see Annexe II).

Simulated virus concentrations in the Artière and Allier rivers were done considering the mixture of waters coming upstream of these points, and neglecting the duration of water transfer on the considered sections of rivers. For the Artière River downstream the discharge of treated wastewater, virus concentration was then equal to:

$$[v,d]_{Art-dw} = \frac{\left([v,d]_{Art-up} \times Q_{w-Art-up}(d)\right) + \left([v,d]_{rww} \times Q_{bp}(d)\right) + \left([v,d]_{tww} \times \left(Q_{tww}(d) - Q_{lag}(d)\right)\right)}{Q_{w-Art-up}(d) + Q_{bp}(d) + Q_{tww}(d) - Q_{lag}(d)} \quad (10)$$

where $[v,d]_{Art-dw}$, $[v,d]_{Art-up}$, $[v,d]_{rww}$ and $[v,d]_{tww}$ are virus concentrations (GC.L^{-1}) on Julian day d in the Artière River downstream wastewater discharge, in the Artière River upstream wastewater discharge, in raw wastewater and in treated wastewater, respectively,

and $Q_{w\text{-}Art\text{-}up}(d)$, $Q_{bp}(d)$, $Q_{tww}(d)$, and $Q_{lag}(d)$ the daily flow on the same day ($m^3.day^{-1}$) of water of the Artière River upstream wastewater discharge, the by-pass, the treated wastewater at the outlet of the treatment plant and the treated wastewater entering the lagoons, respectively. Practically, we neglected $[v,d]_{Art\text{-}up}$. For the Allier River downstream the confluence with the Artière River, virus concentration was then equal to:

$$[v,d]_{All-dw} = \frac{\left([v,d]_{All-up} \times Q_{w-All-up}(d)\right) + \left([v,d]_{Art-dw} \times Q_{Art-dw}(d)\right)}{Q_{w-All-up}(d) + Q_{Art-dw}(d)} \quad (11)$$

where $[v,d]_{All\text{-}dw}$ and $[v,d]_{All\text{-}up}$ are virus concentrations ($GC.L^{-1}$) on Julian day d in the Allier River upstream and downstream the confluence of Artière, respectively, and $Q_{All\text{-}up}(d)$ and $Q_{Art\text{-}dw}(d)$ the daily flow on the same day ($m^3.day^{-1}$) of water of the Allier River upstream the confluence with the Artière River and of the Artière River downstream the discharge of treated wastewater. As we could not simulate daily variations in virus concentration in Allier River upstream the Artière River confluence, we estimated the difference in virus concentration in the Allier River between upstream and downstream for different scenarios of contamination of the Allier River upstream the confluence: zero-contamination, constant high contamination or contamination proportional to that of the Artière river. We then focused on the simulated difference of equivalent virus concentrations between Allier River upstream and downstream the confluence:

$$[v,d]_{All-dw}[v,d]_{All-up} = [v,d]_{All-up} - \frac{\left([v,d]_{All-up} \times Q_{w-All-up}(d)\right) + \left([v,d]_{Art-dw} \times Q_{Art-dw}(d)\right)}{Q_{w-All-up}(d) + Q_{Art-dw}(d)} \quad (12)$$

4. Results and Discussion

a. Acute gastroenteritis and drug consumption; relationship with virus shedding

Over the 1st September 2015 - 31 August 2016 period, the 22 914 declared AGE cases corresponded to probabilities to have been ill and to consult a physician of about 0.096 for all the inhabitants of the wastewater collection basin. This probability depended on the age group in the order "1-4 years">"5-65 years">">65 years" (.

Table II-1). The type of drugs varied with the age group: main differences between them concern young children (1-4 years old) who are first re-hydrated with oral rehydration solutions (ORS). Without taking into account the two ORS groups (26% of the young children prescriptions) and the 'other' group, the corrected ratio for vomitive symptoms concern 67, 82 and 70% of the "<5 years", "5-65 years" and ">65 years" groups suggesting that viral symptoms could have concerned a great proportion of all the age groups. The probabilities of vomiting for each age group in the whole sick population were estimated from the probability of vomiting within the subset of sick people consulting a physician. Values for

the <5 years' group (0.48) and '>65 years' group (0.51) were under the presently used threshold (0.52) to have viral gastroenteritis, whereas its higher value (0.67) for '5-65 years' group lead to an estimate of 100% of viral cases within this group. However, working only on the annual proportion of vomiting symptoms didn't enable to take into account periods during which the proportion of vomiting symptoms exceeds the 0.52 threshold retained in our study for exclusively non-viral gastroenteritis, and this threshold as well as the 0.66 threshold for exclusively viral gastroenteritis are questionable. In addition, the proportion of viral gastroenteritis cases in the < 5 years age class was surely underestimated, as antiemetics are rarely prescribed for young children (Assathiany *et al.*, 2013); in 2008, antiemetics were still prescribed in France for about 75% of children < 9 years having vomiting symptoms (Pfeil *et al.*, 2008).

Table II-1: Statistical data on acute gastroenteritis (AGE) leading to physician consultation and medical prescription as a function of the age group over the September 1st 2015 - August 31st 2016 period.

Age group (years)	**<5**	**5-65**	**>65**	**All ages**
p(AGE and consult)	0.38	0.09	0.04	0.10
$p_{AGE/consult.}$***(antiemetic only)***	0.29	0.43	0.36	0.39
$p_{AGE/consult.}$***(antidiarrheal only)***	0.24	0.17	0.26	0.19
$p_{AGE/consult.}$***(antiemetic & antidiarrheal)***	0.20	0.33	0.25	0.30
$p_{AGE/consult.}$***(ORS <1 year)***	0.02	0	0	0.004
$p_{AGE/consult.}$***(ORS 1-15 years)***	0.24	0.002	0	0.05
$p_{AGE/consult.}$***(other)***	0.02	0.07	0.13	0.07
$p_{AGE/consult.}$***(vomitive symptom)****	0.67	0.82	0.70	0.79
p_{AGE}***(vomitive symptom)*****	0.48	0.67	0.51	0.63
p_{AGE}***(viral infection)******	0.00	1.00	0.00	0.77

: assessed from the sum p_{AGE}(antiemetic only)*+ p_{AGE}*(antiemetic & antidiarrheal)* and ignoring the ORS and 'other' groups.

**: using Equation (2) to estimate the actual proportion of sick people having vomiting symptoms from the proportion within the set of people consulting a physician.

***: assuming that non-viral infection and viral infections lead statistically to 52 and 66% of all prescriptions requiring an antiemetic and/or antidiarrheal.

Seasonal variations in the number of declared cases were described using moving average over 7-day windows (**Figure II.2** and **Figure II.3a**). The total number of reported cases varied with the season, with a minimum of 20.9 new declared cases (based on moving average) per day between June and mid-November 2015 and between mid-April and September 2016, and a maximum of 116 new declared cases per day the rest of the year, i.e. during approximately the winter season (**Figure II.2**). Similarly and taking into account only antiemetic, antidiarrheal, antiemetic+antidiarrheal prescriptions, the proportion of prescriptions with antiemetic increased between mid-August 2015 and mid-February 2016 from approximately 0.6 to nearly 0.9, whereas the proportion of prescribed treatments with antidiarrheal decreased during the same period from a little more than 0.7 to 0.45 (**Figure II.3b**), in partial agreement with viral increase between December and June in Poland (Baumann-Popczyk *et al.*, 2012).

Seasonal variations in the probability of vomiting for the whole population were estimated from seasonal variations in the probability of vomiting within the subset of sick people consulting a physician (**Figure II.3b-c**). From these estimates, we deduced seasonal variations in the probability and the number of viral acute gastroenteritis (**Figure II.4a-c**) with the current proportions of 52% and 66% of acute gastroenteritis with viral and bacterial aetiologies, respectively. Although the results seem very promising, the proportions of emetic symptoms for gastroenteritis of viral or bacterial origin must be re-estimated to better adjust the simulated and experimental viral excretions.

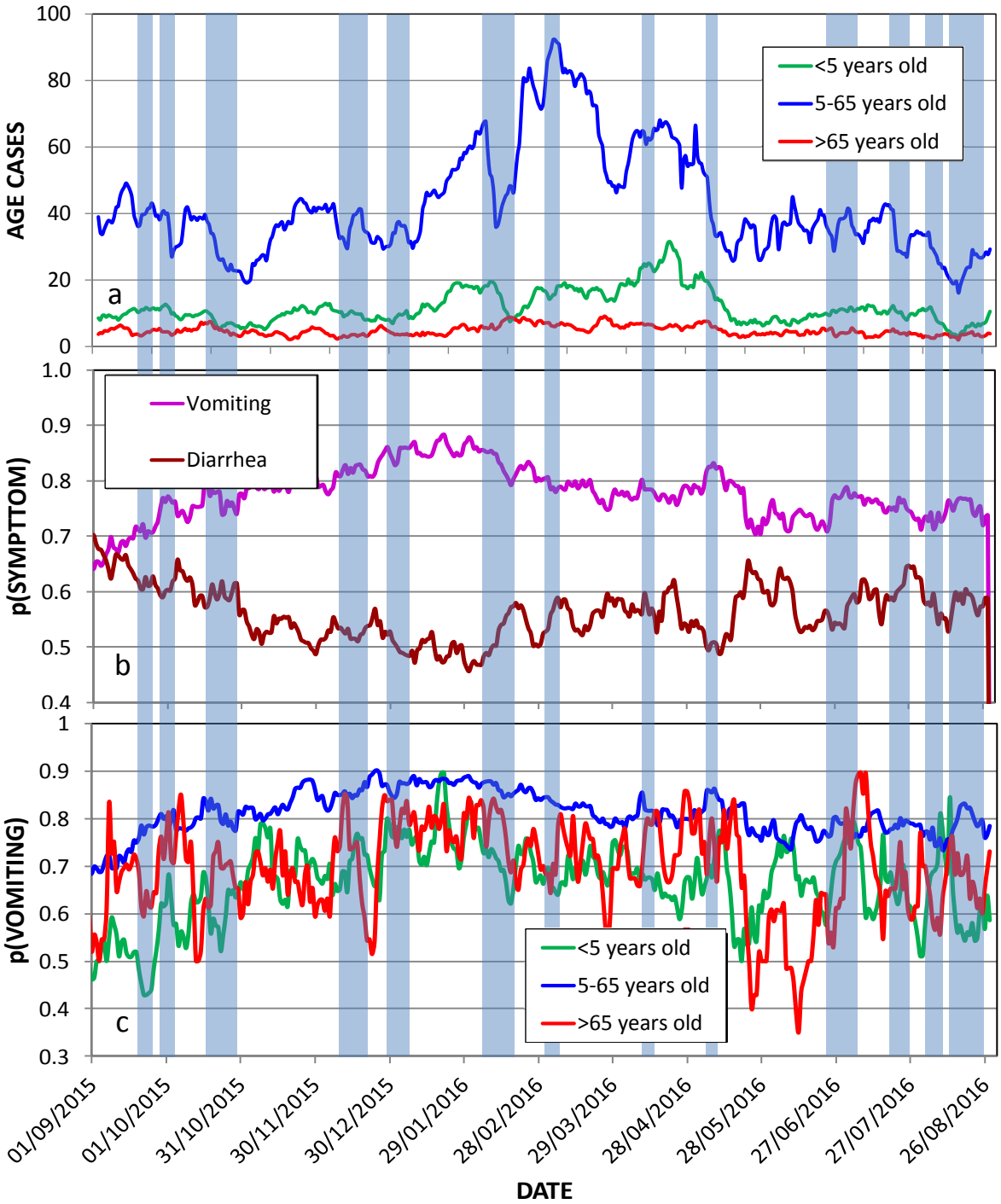

Figure II.3 a-c: Seasonal variations in acute gastroenteritis with medical prescriptions. (a) acute gastroenteritis for each age group; (b) probabilities of vomiting and/or having diarrhea for the all age groups; and (c) probabilities of vomiting for each age group.

(: periods where acute gastroenteritis and/or symptoms trends varies between age groups)

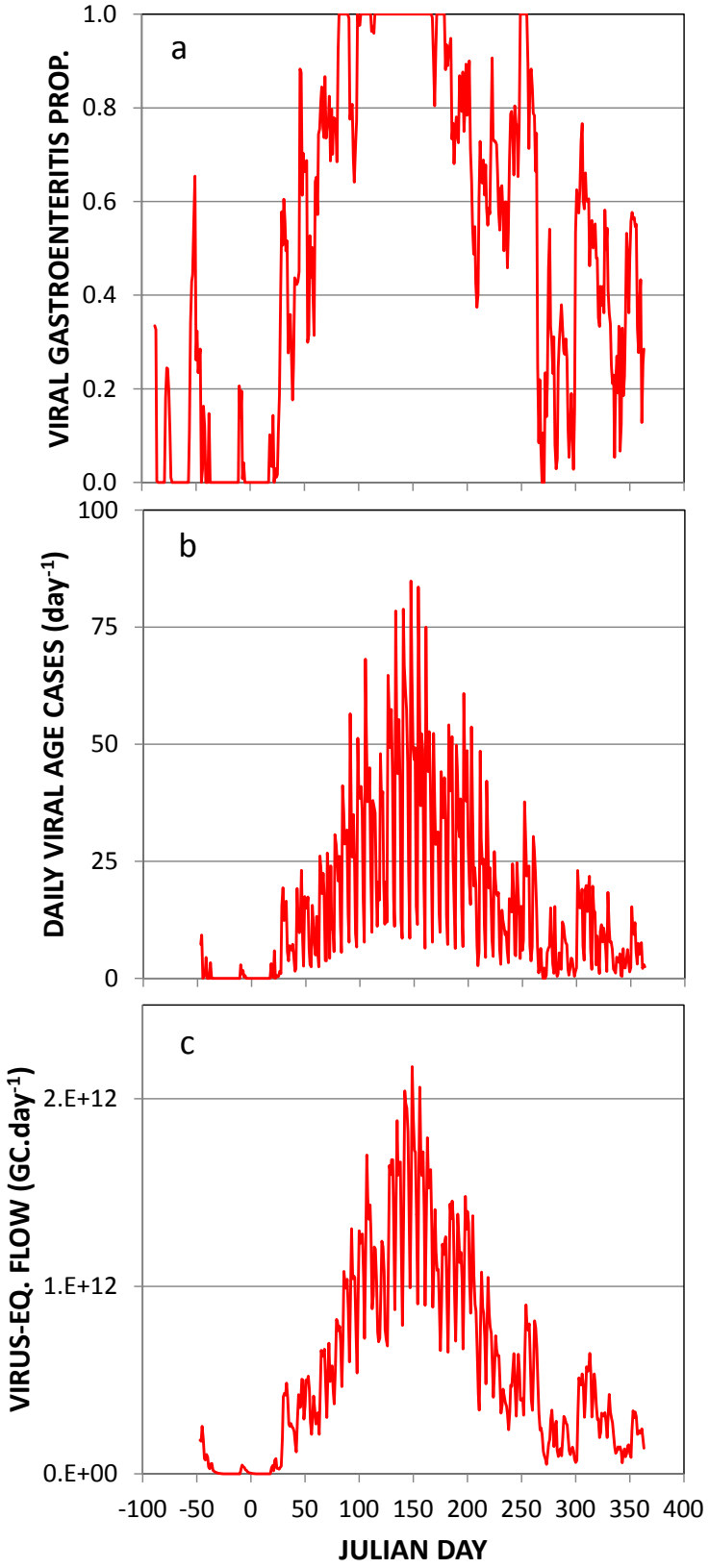

Figure II.4 a-c: Simulation from epidemiologic data of (a) the proportion of viral acute gastroenteritis, (b) the daily number of viral acute gastroenteritis, and (c) the concentration of viruses within raw wastewater at the inlet of the treatment plant. (Day 0: 1st September 2015).

b. Viral contaminations of wastewaters and freshwater bodies; virus flows

Quantified viruses were found almost systematically in raw wastewater with the exception of the hepatitis A virus, which was never detected (**Figure II.5a**). When above their detection thresholds, their concentrations were comprised between $10^{+3.1}$ and $10^{+7.0}$, $10^{+3.8}$ and $10^{+5.8}$, $10^{+5.1}$ and $10^{+7.0}$, $10^{+3.8}$ and $10^{+5.2}$, and $10^{+5.8}$ and $10^{+10.0}$ GC.L^{-1} for noroviruses GI, noroviruses GII, rotaviruses, enteroviruses and adenoviruses, respectively. When they were above their detection thresholds, their concentrations in treated wastewater at the outlet of the wastewater treatment plant (i.e. without additional tertiary treatment in lagoons) were about one to one thousandth times the corresponding values in raw wastewater (**Figure II.5b**). Detection was much less sensitive than that for river viruses due to the initial filtration of 100 L of water. Thus, since treated wastewater discharge contributed for 76% (between 39 and 90%) of the flow of Artière River downstream of their point of discharge, the flow of viruses at the outlet of the wastewater treatment plant was better assessed from the increase in the amount of viruses carried by the Artière River between upstream and downstream of the wastewater discharge point, increased by wastewater withdrawal for filling the lagoons and decreased by virus flow resulting from by-pass.

Virus removal in wastewater treatment plant was estimated by the ratio of the daily virus flow at the inlet of the wastewater treatment plant to the daily flow of viruses at the outlet of the treatment plant (see **Annexe II**). While the Artière River received only treated wastewater from a small treatment plant (120 PE) upstream the discharge by the *'Trois Rivières'* treatment plant, virus concentrations upstream (results not shown) were sometimes of the same order of magnitude as virus concentrations downstream the discharge by the *'Trois Rivières'* treatment plant (**Figure II.5c**), although generally lower. The annual geometric averages of virus removals within the wastewater treatment plant over the annual period 1st September 2015 – 31st August 2016 were 2.4, 1.9, 3.3, 2.6 and 2.5 $\log_{10}$ for the norovirus GI, norovirus GII, rotavirus, enterovirus and adenovirus, respectively. The high variability between estimates led to uncertainties in the seasonal variations (see **Figure S4** in Annexe II); nonetheless, the differences of $\log_{10}$ removals between summer 2015 and winter 2016 were 0.9, 2.7, 1.7 and 2.2 for norovirus GI, norovirus GII, enterovirus and adenovirus, respectively. These orders of magnitude could be explained taking into account variations in wastewater temperature (see **Figure S5** in Annexe II), and a rate of virus immobilization/destruction varying with temperature according to a Q_{10} equation with Q_{10} of about 1.4. However, for simultaneous raw and treated wastewater samplings at about 10 h a.m. U.T., the apparent virus removals exceed the daily averages of about 0.2, 0.3, 0.1 and 0.2 $\log_{10}$ for the considered periods in autumn, winter, spring and summer seasons, respectively (see **Figure S6** in Annexe II).

Virus concentrations in the Allier River upstream the confluence with the Artière River were generally of the same order of magnitude as those in the Artière River at the confluence with the Allier River (**Figure II.5d** and **Figure II.6a-f**); it explained why virus concentrations in the Allier River downstream the confluence with the Artière River could be higher or lower to those upstream the confluence (results not shown). In addition, we noted differences in virus concentrations between the Artière River downstream wastewater discharge and the Artière River just before its confluence with Allier River (**Table II-2**), whereas no significant water intake or removal seems to alter the flow of Artière between these two sites. Viruses were never detected in groundwater of the alluvial aquifer near Allier River, except at 2 dates for the well at 400 m from the Allier bank adenoviruses detected one time, noroviruses GII detected the other time) (results not shown).

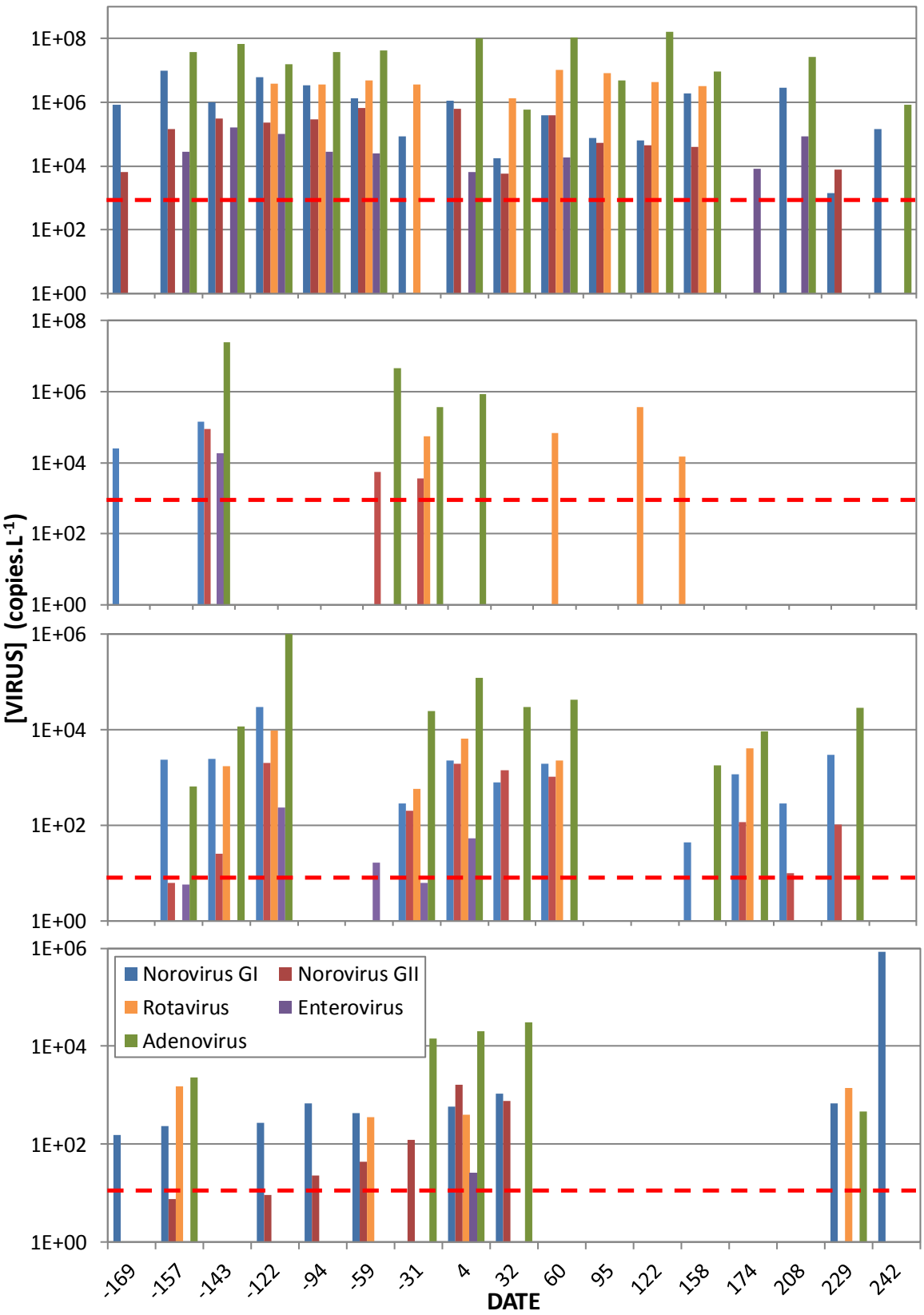

Figure II.5 a-d: Measured virus concentrations in (a) raw wastewaters, (b) treated wastewater at the outlet of the treatment plant, (c) in the Artière River downstream treated wastewater discharge, and (d) in the Allier River upstream the confluence with the Artière River.

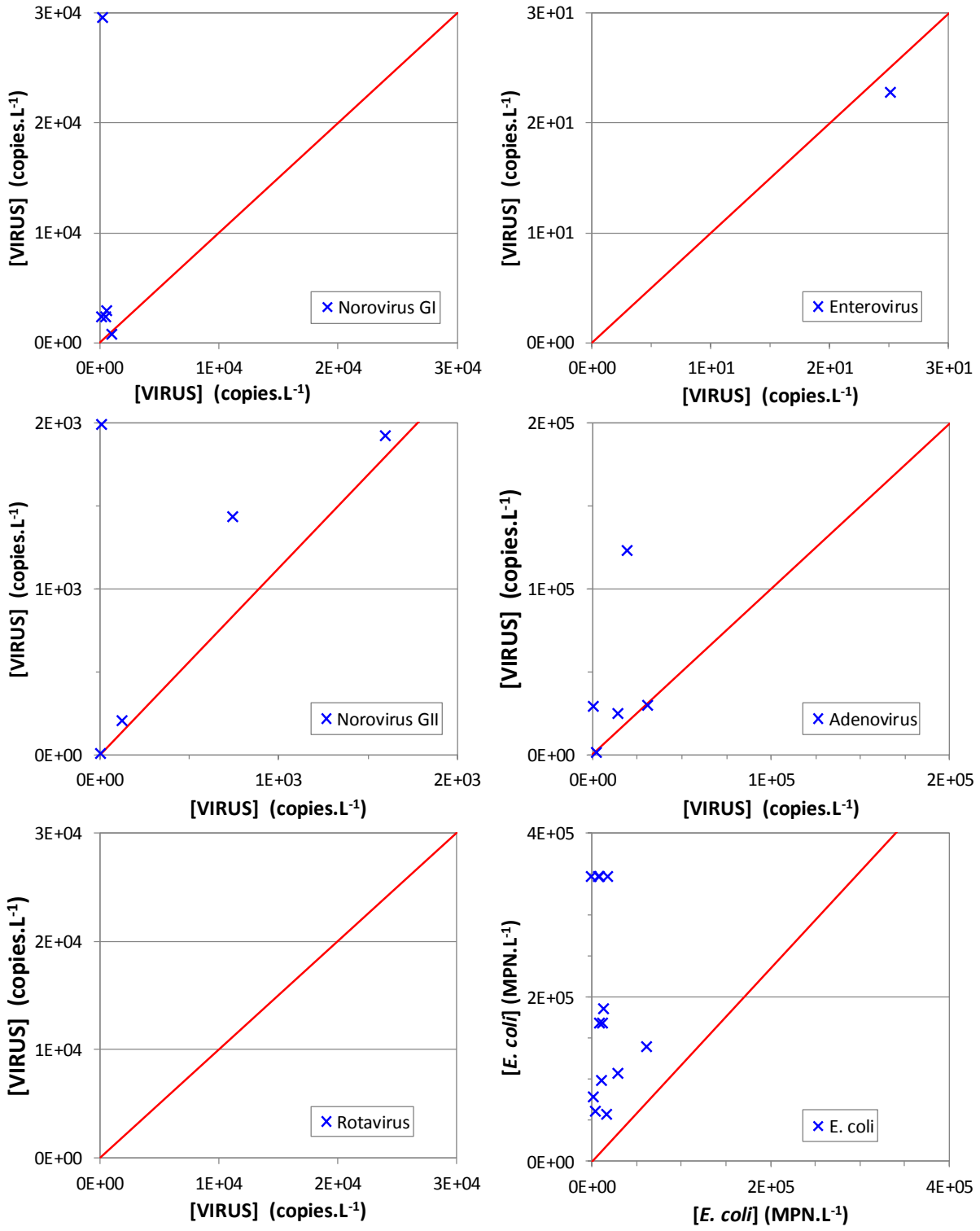

Figure II.6 a-f: Concentrations in the Artière River downstream wastewater discharge (x-axis) vs concentrations in the Allier River upstream the confluence with the Artière River (y-axis) of (a) noroviruses GI, (b) noroviruses GII, (c) rotaviruses, (d) enteroviruses, (e) adenovirus, and (f) *Escherichia coli*.

Table II-2 : Ratios of concentrations in the Artière River downstream wastewater discharge to Artière River near the confluence with Allier River. (- ratio not calculated when viruses were not detected either downstream wastewater discharge or near the confluence with the Allier River).

Date	Nov GI	Nov GII	RoV	EnV	AdV	*E. coli*
09/29/2015	-	-	-	-	-	0.68
11/03/2015	-	-	-	-	-	29.2
12/01/2015	0.75	0.51	1.36	-	0.39	0.23
01/05/2016	1.16	0.01	0.22	-	0.03	1.34
02/02/2016	17.9	0.10	-	-	0.51	0.58
03/01/2016	-	-	-	-	-	0.44
04/05/2016	-	-	-	-	-	1.00
05/02/2016	-	-	-	-	-	0.30
06/07/2016	1.62	-	-	-	0.43	0.18
06/23/2016	-	-	-	-	-	0.09
07/27/2016	276	9.83	-	-	-	0.18
08/17/2016	2790	106	-	-	39.5	0.06
08/30/2016	-	-	-	-	-	0.22

Although they couldn't be considered as a conservative tracer, viruses and *Escherichia coli* concentrations could *a priori* provide additional information on water flow conservation. However:

- in the Artière River, the ratios of concentrations from sampling downstream wastewater discharge to sampling near the confluence with Allier River and their temporal variations greatly varied between dates and microbial entities (**Table II-2**). We could not correlate these variations with possible rainfalls in the days preceding sampling, especially for low ratio values (results not shown); high values could partly result from unidentified additional virus and E. coli sources;

Table II-3 : Ratio of Virus or E. coli flows in the Allier River downstream the confluence with the Artière River to the sum of Virus or E. coli flows in the Allier River upstream the confluence and in the Artière River downstream wastewater discharge. (- ratio not calculated when viruses were not detected in one of these compartments).

Date	Nov GI	Nov GII	RoV	EnV	AdV	*E. coli*
07/16/2015	-	-	-	-	-	
07/28/2015	-	-	-	-	-	
08/11/2015	-	-	-	-	-	
09/01/2015	0.20	0.12	-	-	-	
09/29/2015	-	-	-	-	-	0.71
11/03/2015	-	-	-	-	-	0.36
12/01/2015	-	-	-	-	0.03	0.58
01/05/2016	-	0.72	-	0.89	1.05	0.47
02/02/2016	0.09	0.19	-	-	0.92	1.19
03/01/2016	-	-	-	-	-	0.99
04/05/2016	-	-	-	-	-	2.27
05/02/2016	-	-	-	-	-	0.80
06/07/2016	-	-	-	-	-	2.32
06/23/2016	-	-	-	-	-	0.90
07/27/2016	-	-	-	-	-	-
08/17/2016	1.46	-	-	-	-	0.25
08/30/2016	-	-	-	-	-	1.19

- at the confluence between the Artière River and the Allier River, the ratio of the downstream flow on the Allier River to the sum of upstream flows on the Artière and Allier rivers in terms of viruses and E. coli varied greatly (**Table II-3**). These variations can perhaps be explained by sampling in the Allier River near the banks in water that is not very mobile and by the uncertainties in the quantities of virus measured.

c. Flow of viruses; fitting simulations based on epidemiology and shedding to estimates based on measurements.

Simulated total daily virus shedding based on epidemiologic data were compared to the measured flow of viruses at the inlet of the wastewater treatment plant after fitting experimental equivalent virus concentrations defined in equation (9) to simulated ones. By doing this, we assumed no immobilization or destruction of viruses in the sewer system. There was a good agreement between simulated trends and weighted observations with occasional large deviations (**Figure II.7**).

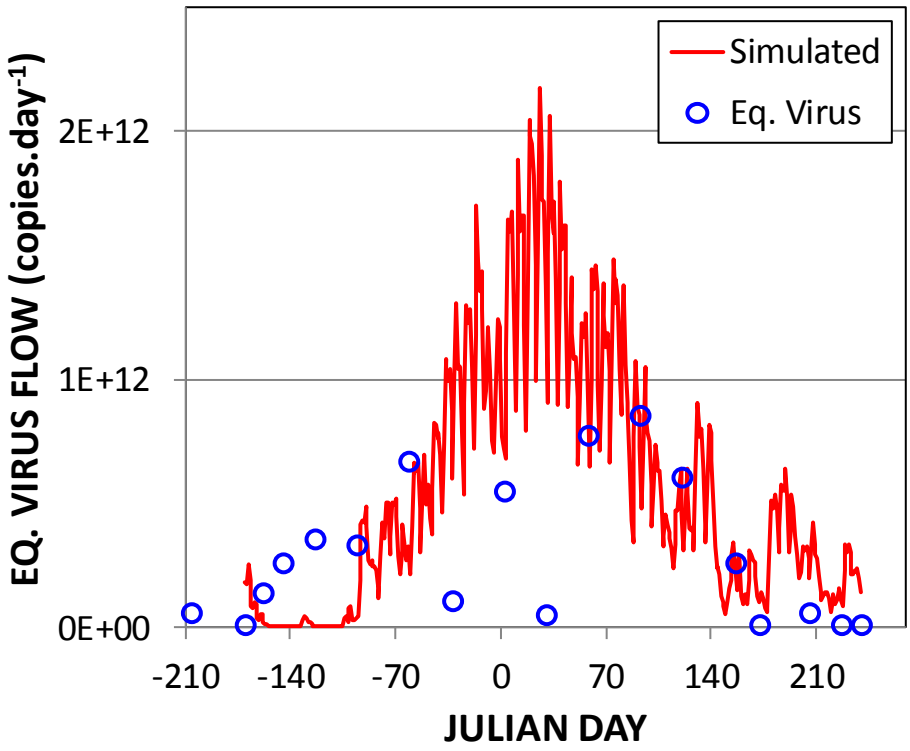

Figure II.7 : Comparisons of simulated total daily virus shedding based on epidemiologic data to experimental equivalent virus concentrations assuming their following expression $[v_{eq}, d] = 10^{-2} \times [NoGII, d] + 5.8\ 10^{-4} \times [RoV, d] + 4.6\ 10^{-5} \times [AdV, d]$ (Day 0: 1st September 2015).

It is important to note that measurements performed by qPCR have high uncertainties, making their use more appropriate for variations over several powers of 10 than for variations of viral acute gastroenteritis numbers of less than 10 between winter and summer. Estimations of the weighting coefficients were: a=0.0, b=1 10^{-2}, c=5.8 10^{-4}, d=0.0 and e=4.6 10^{-5} for noroviruses GI, noroviruses GII, rotaviruses, enteroviruses and adenoviruses, respectively. While absolute values depend on the value of C_{max} chosen in equation (5) and should not be discussed, values relative to each other are indicative of virus specie contributions per virion

to acute gastroenteritis. *b* was clearly the highest weighting coefficient, indicating the important contribution of noroviruses GII to acute gastroenteritis. By contrast, the zero *a* and *d* values suggest that noroviruses GI and enteroviruses did not greatly impact the number of acute gastroenteritis cases.

By combining virus removal in the wastewater treatment plant (Annexe II) with the mixture of water coming from the Artière River upstream the wastewater discharge, treated wastewater discharged into the Artière River (i.e. not deviated towards the lagoons) and raw wastewater discharged without treatment using the by-pass of the treatment plant, we simulated virus concentrations in the Artière River downstream wastewater discharge (**Figure II.8**). These values were compared to equivalent virus concentrations estimated from the combination of measured virus concentrations as proposed in equation (9) using the weighting coefficient values already estimated from virus flow in raw wastewaters. There was a good agreement between simulated trends and weighted observations, with the exception of a very high weighted observation on September 1, 2015 and 2 consecutive zero values between early April 2016 and early May 2016 (**Figure II.8**). A bypass of 320 m^3 was operated on September 1st, 2015, the day when the weighted observations corresponded to a concentration in equivalent viruses (6 10^{+5} viruses.L^{-1}) significantly higher than the value simulated from epidemiologic data (1.9 10^{+2} viruses.L^{-1}). At this date, it is likely that the by-pass (very low compared to other dates) was operated during less than 1 hour concomitantly to our water sampling leading to a punctual contamination of the Artière River higher than the simulated value assuming that the by-pass was operated over 24 hours. Two other peaks reaching about 1.5 10^{+6} virus.L^{-1} were simulated by using epidemiologic data on 2 other days with a daily by-pass that could reach 10 500 m^3.day^{-1} (more than 30 times the by-pass flow of the 1st September 2015). Beyond this punctual difference, the simulated results show us how much the operation of the by-pass can occasionally (but recurrently) affect the contamination of the Artière River (**Figure II.8**).

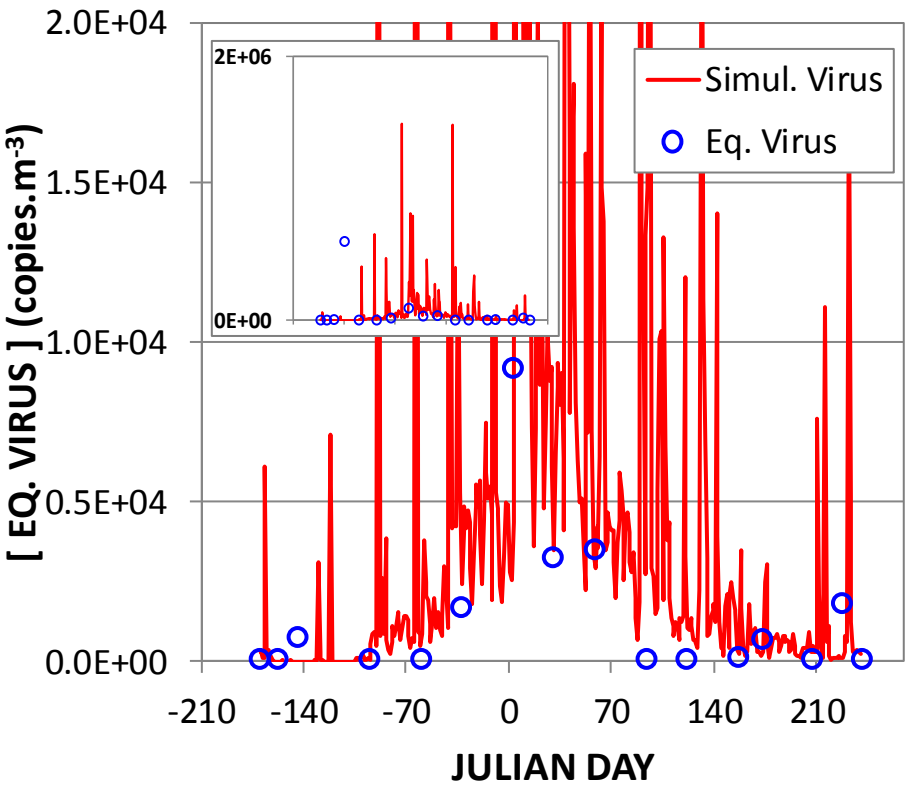

Figure II.8 : Comparisons of simulated virus concentrations in the Artière River downstream the discharge of wastewater, when neglecting the virus contamination of the Artière River upstream the wastewater discharge, to the sum of weighted measured virus concentrations. (Virus contamination of the Artière River upstream wastewater discharge could have increased simulated equivalent virus concentrations between about 9 10^{+1} and 1 10^{+3} copies.L^{-1}, except the 1st September 2015 where the increase in simulated value reached 7 10^{+4} copies.L^{-1}). (Day 0: 1st September 2015).

Differences in virus concentrations in the Allier River between the upstream and downstream of the confluence with the Artière River were simulated according to 3 scenarios of virus contamination of the Allier River upstream the confluence (**Figure II.9**). Except for the unrealistic scenario 1 of zero contamination of the Allier River upstream the confluence, simulations showed that the Artière could sometimes dilute the virus contamination of the Allier River or increase this pollution.

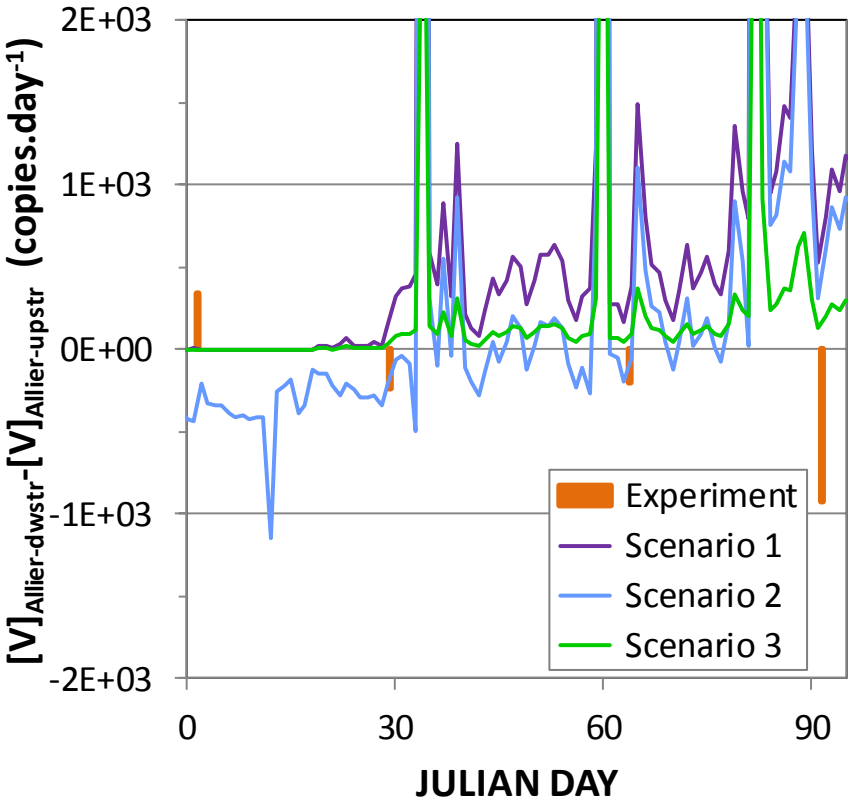

Figure II.9 : Simulated differences in equivalent virus concentrations in the Allier River between downstream and upstream the confluence with the Artière River for three scenarios of Allier viral contamination upstream the confluence: scenario 1: zero pollution; scenario 2: 10^{+4} copies.L^{-1}; scenario 3; equivalent virus concentration equal to 0.75 times the concentration in the Artière River downstream wastewater discharge.

5. Conclusions

The objectives of this work were to characterize throughout the year viral contaminations of wastewaters and of surface and underground freshwaters potentially affected by these wastewaters, and to check whether such variations could be simulated by a model taking into account human viral shedding indirectly estimated from epidemiological data, viral removal in *Clermont-Auvergne-Métropole* WWTP and dilutions in rivers.

Limits in the epidemiological analysis resulted from our working assumptions, including the proportion of vomiting symptoms estimated without taking into account ORS and 'others' prescriptions, and the retained proportions of vomiting symptoms for viral and non-viral acute gastroenteritis (0.66 and 0.52, respectively, issued from Arena et al. (2014), whereas these value correspond to cases respectively with and without viruses in stools). In addition, such an approach is all the more valid as the population is large, while the small size of daily newly infected populations may lead to deviations from the estimated values. Temporal trends in the estimated viral acute gastroenteritis total number were consistent with

the seasonality of viral acute gastroenteritis (Baumann-Popczyk *et al.*, 2012; Prevost *et al.*, 2015b). Periods during which variations in new declared acute gastroenteritis cases and/or their vomiting symptoms differed between age classes offered the possibility to make hypotheses on the contamination routes during these periods (e.g. oyster consumption during the end of the year holidays by 5-64 years population). It is likely that simultaneous access to mucous diarrhea, fever data, which also statistically distinguish viral etiology from bacterial ones, would have improved the estimate of the proportion of viral gastroenteritis cases.

Limits in virus experimental quantifications resulted from uncertainties and detection thresholds of qPCR, and other viruses that were not quantified (e.g. Aichivirus; Astrovirus; hepatitis E virus) (Prevost *et al.*, 2015b). Raw wastewaters were contaminated year-round with variations of 1-2 $\log_{10}$ for the detected values. The removals (2-3 $\log_{10}$) in the wastewater treatment plant were relatively high, and caused by the long residence time in the treatment plant (2-3 days), the anoxic zone of the activated sludge basin, and the use of iron chlorosulfate to precipitate phosphates. Virus contaminations were diluted in the rivers; the Artière River could contaminate the Allier River or dilute its virus load. Viruses were never detected in the last lagoon of the *'Sucrerie de Bourdon'*, the drilling of vegetable gardens near the Artière River bank, and the alluvial aquifer in contact with the Allier River.

Limits in the modelling approach of virus shedding and fate in the wastewater treatment plant and rivers resulted from working assumptions. They include the removal of viruses in this sewer system that was neglected, water sources and sinks in the Artière River that were ignored, virus shedding according to Teunis et al. (2015) median shedding, and various parameter values (average water residence time in the sewer system, constants used to estimate the actual number of acute viral gastroenteritis). As our model allowed simulating the virus flow without distinction of species, simulated data were compared to the weighted sum of experimental ones. By doing this, it was possible to simulate the equivalent virus load of raw wastewaters at the inlet of wastewater treatment plants as well as virus concentration in the Artière River downstream wastewater discharge. Beyond this, the model predicts by-pass impact, with virus concentrations up to 20 times higher than virus concentrations without by-pass. Unfortunately, large uncertainties remain in measurements as well as in the comparison of measurements and simulations.

Despite the uncertainties regarding certain aspects of this work, two types of finalized benefits can be envisaged:

- first, assuming that the time between onset of symptoms and physician consultation is close to the average residence time of raw wastewaters in the sewer system, real-time access to data from physician consultation and drug purchase would provide early access to a simulated evaluation of the amount of virus arriving at the wastewater treatment plant. One can imagine that such information would then allow real time adaptation of additional tertiary treatment, like using 254 nm UV, all the more that the mean residence time of wastewaters in the *'Trois Rivières'* wastewater treatment plant is about 2.5-3 days. However, virus equivalent concentrations vary only by a multiplication factor of about 10,

and raise the question of the relevance of customization of treatments to the most contaminated waters;

- second, it is possible to assess the impact of water management scenarios alternative to the ones presently used: (i) all treated wastewater discharged in the Artière River, and (ii) a constant daily wastewater withdrawal of 50% of all treated wastewaters for filling additional water reservoirs (**Figure II.10**). During winter (**Figure II.10a** for September), the present management of wastewater is equivalent to the environmental discharge of all wastewaters, and the 50% wastewater withdrawal scenario generally leads to lower virus concentration. In summer (**Figure II.10b** for July), generally the current management scenario is the least polluting scenario as the proportion of treated wastewater withdrawal of exceeded 50% of all treated wastewater. In by-pass periods, the two scenarios with wastewater withdrawal could lead to increased virus concentration in the Artière River as the contamination due to the by-pass was then diluted in a lower volume of water.

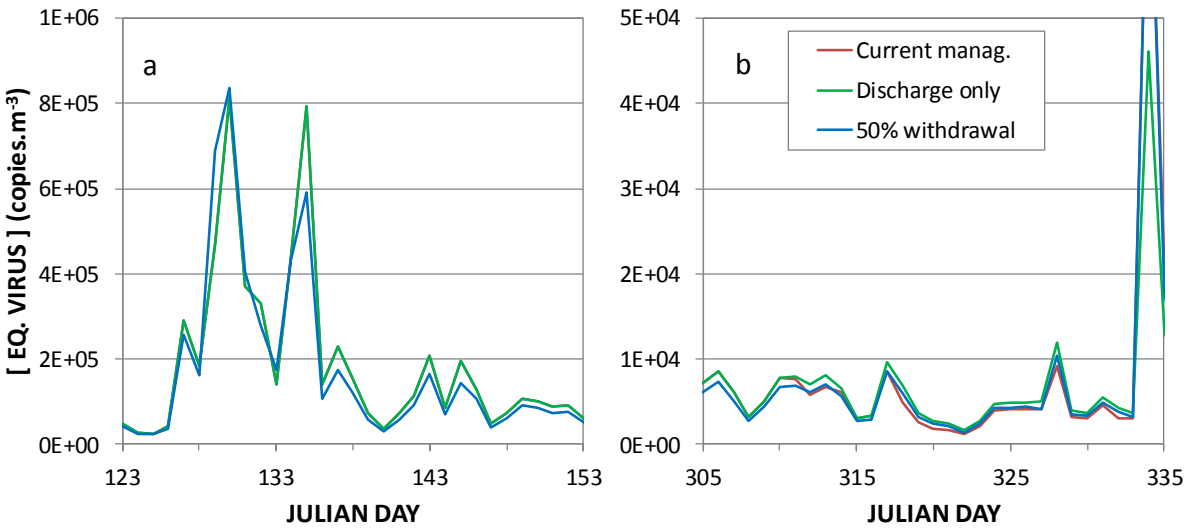

Figure II.10 : Comparison of the simulated impacts of treated wastewater management for January and July 2015 months according to the three following scenarios: (i) treated wastewater discharged entirely in the Artière River, (ii) present withdrawal of treated wastewater operated for the *'ASA Limagne Noire'* association, and (iii) daily withdrawal of 50% of treated wastewater to fill reservoirs throughout the year.

Chapitre III

Proposition et évaluation d'un modèle d'élimination et d'immobilisation réversible des norovirus murins dans un phaeozem calcique pour diverses conditions de contamination et de rinçage

Ce chapitre a donné lieu à la soumission au journal *European Journal of Soil Science* d'un article intitulé *"Modelling the removal and reversible immobilization of murine noroviruses in a phaeozem under various contamination and rinsing conditions"*, cosigné par Tesson V., de Rougemont A., Capowiez L., Renault P..

Les virus entériques apportés au sol par des eaux d'irrigation peuvent contaminer les eaux souterraines ou être internalisés dans les plantes *via* leurs racines et migrer vers des parties consommées crues sans être inactivés. Dans le sol le devenir des virus dépend du virus, du sol et de la solution du sol. Notre objectif était de proposer et évaluer un modèle de devenir du norovirus murin (modèle du norovirus humain) dans un phaeozem calcique, sol bien représenté en Limagne Noire.

Nous avons étudié l'abattement du norovirus murin et son immobilisation réversible dans des colonnes d'agrégats en fonction de la procédure de saturation (sous vide partiel ou non), des conditions entre la période de contamination et la période propice à un rinçage (niveau de dessiccation et temps d'attente), de la température et de la composition chimique de la solution circulante. Les virus ont été quantifiés avant et après une filtration à 0,45 µm par RT-qPCR et isolement sur culture cellulaire. Les résultats expérimentaux vérifient un modèle combinant le transport des virus sous forme libre et colloïdale, l'abattement viral et l'adsorption réversible sur les colloïdes en suspension, la surface des agrégats et la phase solide au sein des agrégats. A 20°C, dans le cas d'une contamination de 1 à 7 jours par une solution artificielle de sol contaminée suivie d'un rinçage de 7 heure, le devenir des virus a pu être décrit en combinant un abattement journalier de 0.38 $\log_{10}$ et une faible immobilisation réversible décrite par une isotherme de Freundlich (kF = 102.2, nF = 1.53) exprimant la faiblesse de l'adsorption réversible des virus libres dans l'eau mobile. Un séchage partiel des agrégats sans désaturation n'a pas affecté la remobilisation des virus immobilisés réversiblement. Un enrichissement en Mg^{2+} a induit des changements géochimiques en partie temporaires, avec dix fois plus de virus adsorbés sur les colloïdes en suspension que sous forme libre, et une adsorption importante des virus sur la surface extérieure des agrégats. De la même façon, la composition de l'eau souterraine favorisait la remobilisation du norovirus murin.

Pour alimenter de façon pertinente une évaluation quantitative des risques associée à une irrigation agricole par des eaux usées, ce travail devrait être couplé (i) à un volet atmosphérique (devenir atmosphérique pour les virus n'atteignant pas le sol (Teltsch & Katzenelson, 1978)ou la plante pendant l'irrigation ; aérosolisation des virus préalablement déposés à la surface des plantes ou du sol (Girardin *et al.*, 2016)), (ii) à un volet contamination des plantes par internalisation à partir des racines (Renault *et al.*, 2017), et (iii) éventuellement à un risque d'atteinte de l'aquifère sous-jacents (Borchardt *et al.*, 2007; Hunt & Johnson, 2017). Le travail sur le devenir des virus entériques dans le sol est plus directement relié à ces deux derniers volets. De fait, nous avons-nous-même participé à un travail ponctuel sur la contamination d'oignons verts cultivés sur le même sol (Renault *et al.*, 2017)(cf. Annexe I). On pourrait imaginer que le temps de résidence moyen de l'eau puisse servir à évaluer un abattement en virus des eaux absorbées par les racines. Toutefois, on peut imaginer que les zones absorbantes soient parfois atteintes très rapidement par des eaux s'écoulant rapidement (un peu comme le by-pass des stations d'épuration) et modifier l'évaluation réelle des quantités de virus entrant dans la plante. Au-delà, le devenir des virus dans la plante reste un sujet d'étude important avec en questions prioritaires (i)

l'immobilisation importante ou non dans les vaisseaux du métaxylème, (ii) l'accumulation éventuelle des virus à l'extrémité du métaxylème, et (iii) l'excrétion éventuelle des virus à l'extérieur des parties aériennes avec la transpiration ou la guttation de la plante.

1. Summary

Enteric viruses entering the soil with contaminated irrigation water can reach groundwater or be internalized in plants *via* their roots without being inactivated. Their fate in the soil depends on the virus, the soil and the soil solution. In order to write a mathematical model suitable for a calcaric phaeozem, we investigated murine norovirus removal and reversible immobilization in aggregate columns according to saturation procedure, conditions between times of contamination and rinsing, temperature, and soil solution. Viruses were quantified before and after 0.45 µm filtration using RT-qPCR and plaque assays. Experimental results supported a model combining free and colloidal transport of viruses in mobile water, exchange of free viruses between mobile and immobile waters, virus removal, and reversible virus adsorptions on suspended colloids, aggregate surface and particles within aggregates. For artificial soil solution at 20°C, the fate of viruses in contaminations lasting 1 to 7 days followed by 7 hours rinsing could be described by combining 0.38 log10 daily removal and weak reversible immobilization using a Freundlich isotherm (kF = 102.2, nF = 1.53), which explained why free viruses prevailed in mobile water. Partial drying without aggregate desaturation did not affect virus recovery. Mg2+ enrichment induced geochemical changes that faded over time, resulting in up to ten times more viruses adsorbed on suspended colloids than free, and enhanced adsorption on aggregate outer surfaces; similarly, groundwater slowed remobilization. The fate of murine norovirus within a calcaric phaeozem can be described by a model that takes into account geochemical fluctuations.

Key words: wastewater reuse, enteric virus, soil, virus fate, geochemical changes, simulation

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Highlights

1. How far does soil reduce the risk that noroviruses brought by irrigation reach groundwater or roots?
2. Virus removal over a five-day average residence time in phaeozem is about two $\log_{10}$.
3. Reversible virus immobilization is weak; Mg intake favours it, together with colloidal transport.
4. Virus removal and reversible adsorption may be modelled to better assess the risk of contamination.

2. Introduction

The world is facing increasing water scarcity and a decline in freshwater quality (Kumar, 2015). Wastewater reuse for crop irrigation can help to address these problems by upgrading marginal fertilizing resources and stopping their discharge in freshwater bodies (Jaramillo & Restrepo, 2017; Navarro *et al.*, 2015). However, their chemical and microbial contents simultaneously imply environmental, agricultural and health risks (Girardin *et al.*, 2016; Qu *et al.*, 2016). Domestic wastewaters generally contain human enteric viruses (Iaconelli *et al.*, 2016; Jaramillo & Restrepo, 2017; Prevost *et al.*, 2015b) that have often been identified as causing waterborne and foodborne disease outbreaks in industrialized countries (Kirk *et al.*, 2015; Rodríguez-Lázaro *et al.*, 2012; WHO, 2015), and sometimes airborne outbreaks in indoor conditions (Nazaroff, 2011). Noroviruses, reportedly responsible for 36% and 67% of gastroenteritis in the world and in Europe, respectively (Kirk *et al.*, 2015), are by far the leading cause of outbreaks associated with the consumption of leafy vegetables in the USA (Herman *et al.*, 2015). Their environmental transmission is favoured by strong excretion over weeks in sick and healthy carriers (Teunis *et al.*, 2015), resistance to water treatments (Zhou *et al.*, 2015), persistence without inactivation in the environment for several weeks to months (Jaramillo & Restrepo, 2017; Kotwal & Cannon, 2014), and low infectious dose (Atmar *et al.*, 2014).

During irrigation by domestic wastewater, human enteric viruses can be dispersed as bioaerosols or deposited at the surface of the soil and plants (Girardin *et al.*, 2016).Viruses can then disseminate from the soil surface to the aquifer (Murphy *et al.*, 2017) or be internalized in plants *via* their roots and then disseminate to their edible portions without being inactivated (Carducci *et al.*, 2015; DiCaprio *et al.*, 2015; Hirneisen & Kniel, 2013a, 2013c). In the soil, virus fate combines transport, immobilization and inactivation (Bhattacharjee *et al.*, 2002; Dowd *et al.*, 1998; Zhao *et al.*, 2008). These processes are interdependent: viruses are transferred either free or adsorbed on colloids (Syngouna & Chrysikopoulos, 2010), and virus inactivation may be slowed by their immobilization in biofilms (Skraber *et al.*, 2005) or accelerated by their adsorption on metal oxides at solution pH close to neutrality (Warnes *et al.*, 2015; Zhuang & Jin, 2008).

Virus immobilization depends (i) on the species, strain and conformation of capsid proteins (Dowd *et al.*, 1998; Michen & Graule, 2010; Taylor *et al.*, 1981), (ii) on soil minerals and organic compounds (Bradley *et al.*, 2011; Zhuang & Jin, 2003, 2008), and (iii) on the soil solution (Cao *et al.*, 2010; da Silva *et al.*, 2011; Dowd *et al.*, 1998; Taylor *et al.*, 1981). When they are small enough, like noroviruses (27 nm), in contrast to bacteriophage PM2 (60 nm), and not aggregated by pH near the virus isoelectric point (Michen & Graule, 2010), their immobilization results from adsorption rather than trapping in pore constrictions or surface anfractuosities (da Silva *et al.*, 2011; Dowd *et al.*, 1998). Forces involved in virus adsorption include Van der Waals, electrical, hydrophobic and Born forces (Park & Kim, 2015). Whereas Van der Waals forces are always attractive, the repulsive or attractiveness of the electrostatic forces depends on virus and soil particle charges which vary with the pH and the

ionic composition of the solution (Cao *et al.*, 2010; Chattopadhyay & Puls, 2000). A few solids, such as Fe oxides, have high isoelectric points that favour virus adsorption over a wider pH range (Syngouna & Chrysikopoulos, 2010). Adsorption is favoured by the presence of divalent cations (Cao *et al.*, 2010; da Silva *et al.*, 2011; Wong *et al.*, 2012b). Soluble organic compounds may compete with viruses for adsorption (Cao *et al.*, 2010), while solid organic compounds may increase sites on which viruses may be immobilized (Schijven & Hassanizadeh, 2000). Hydrophobic forces are often assumed to result from Lewis acid base interactions (van Oss, 1993) and significantly affect overall forces (Chrysikopoulos & Syngouna, 2012) ; they vary with viruses (Dika *et al.*, 2013), and are reported to increase with temperature (Syngouna & Chrysikopoulos, 2010).

Although some models of virus immobilization in soil have been part of more integrative models of virus fate in soil or the vadoze zone with only cursory experimental validations if any (Bhattacharjee *et al.*, 2002; Yates & Ouyang, 1992a), other models are supported by laboratory experiments on immobilization (Lance & Gerba, 1984; Sadeghi *et al.*, 2013; Sasidharan *et al.*, 2017; Schijven & Šimůnek, 2002) (**Table III-1**). Moving viruses have been assumed to be either exclusively free (Sasidharan *et al.*, 2017; Zhao *et al.*, 2008) or adsorbed on dispersed colloids (Burge & Enkiri, 1978; Mayotte *et al.*, 2017) ; in this last case, inert colloid immobilization was assumed to results mainly from filtration (Yao *et al.*, 1971). Virus immobilization has sometimes been held to be irreversible (Praetorius *et al.*, 2014; Sen, 2011) as suggested by the colloid filtration theory (Yao *et al.*, 1971) and the extended DLVO theory if the distance between viruses and soil particles corresponds to the primary minimum of energy (Praetorius *et al.*, 2014). More often, virus immobilization has been held to be reversible (Cao *et al.*, 2010; Sadeghi *et al.*, 2013; Sasidharan *et al.*, 2017; Tufenkji & Elimelech, 2004) and explained either by a distance between virus and soil particle corresponding to the secondary minimum of energy when this minimum exists (Sadeghi *et al.*, 2013), or by geochemical changes that modify energy profile (Cao *et al.*, 2010). Simultaneous reversible and irreversible immobilizations have also been described by distinguishing adsorption sites (Cao *et al.*, 2010) or simulating the kinetic inactivation of reversibly adsorbed viruses (Tufenkji & Elimelech, 2004). Reversible virus adsorption have been kinetically described by introducing attachment and detachment kinetic coefficients (Sasidharan *et al.*, 2017), other works have described equilibrium between viruses in solution and viruses adsorbed on solids with partition coefficient K_D (Syngouna & Chrysikopoulos, 2010), Freundlich isotherms involving K_F and n_F parameters (Moore *et al.*, 1981a; Vilker & Burge, 1980), and Langmuir isotherms involving $S_{r\text{-}max}$ and β parameters (Grant *et al.*, 1993; Vilker & Burge, 1980). The extended DLVO theory allows them to explain variations in adsorption with context changes (Tufenkji & Elimelech, 2004).

However, experimental works have not sufficiently explored "virus-soil-soil solution-climate" contexts for generic modelling, including unsaturated soil conditions with possible virus adsorption at 'air-water' interfaces (Chu *et al.*, 2001) and the reversibility of the adsorptions after exposure to dry conditions (Lance *et al.*, 1976), especially for real soils containing more than 35% clays. The reversibility of adsorptions in saturated soils must also

be better assessed and, where it exists, the need to describe independently virus adsorption and desorption or the possibility of using only a partition coefficient between the solution and soil solids.

Table III-1 : Main features of reversible immobilization models retained in published papers.

References	Reversible adsorption				Irreversible adsorption	
	Kinetic approach	K_D partition coefficient	Freundlich isotherm	Langmuir isotherm	From soil solution	From reversibly adsorbed pool
Burge & Enkiri (1978)			●			●
LaBelle & Gerba (1979)			●		●	
Vilker & Burge (1980)				●	●	
Moore et al. (1981b)			●			●
Tim & Mostaghimi (1991) with experimental results from Lance & Gerba (1984)	●	●				●
Yates & Ouyang (1992b) with experimental results from Grosser (1985) ; Ungs et al. (1985); Grondin (1987) ; Yates et al. (1988) ; Bales *et al.* (1989) ; Ouyang (1990)		●			●	
Grant et al. (1993)	●			●		●
Powelson & Gerba (1994)		●	●		●	
Dowd et al. (1998)	●	●			●	
Thompson et al. (1998)			●			●
Sim & Chrysikopoulos (1999) with experimental results from Hurst et al. (1980)		●			●	
Gantzer et al. (2001)			●		●	
Schijven & Šimůnek (2002) with experimental results from Schijven et al. (1999, 2000)	●				●	
Flynn et al. (2004)	●				●	
Torkzaban et al. (2006)		●			●	
Cheng et al. (2007)		●			●	
Zhao et al. (2008)			●		●	
Zhuang & Jin (2008)		●			●	
Anders & Chrysikopoulos (2009)		●			●	
Cao et al. (2010)	●					●
Syngouna & Chrysikopoulos (2010)		●			●	
Chrysikopoulos & Syngouna (2012)			●		●	
Sadeghi et al. (2013)	●					●
Chrysikopoulos & Aravantinou (2014)			●		●	
Mayotte et al. (2017)	●					●
Sasidharan et al. (2017)	●				●	
Our upcoming study			●			●

Our objectives were (i) to extend experimental data to a calcaric phaeozem (FAO classification), including the effect of the circulating solution composition and temperature, (ii) to identify the adequate mathematical formalism, i.e. considering kinetic exchanges or only equilibrium between suspended and reversibly adsorbed viruses and then the relevant mathematical formalism (partition coefficient, Freundlich isotherms or Langmuir Isotherms), and check whether variations in associated parameters conform to the extended DLVO theory (Henry, 2017; Tufenkji & Elimelech, 2005; Yao *et al.*, 1971).

3. Materials and Methods

a. Virus, soil and artificial soil solution

The cytopathic CW1 strain of murine norovirus (MNV-1), as surrogate of human enteric noroviruses, was kindly provided by Prof. H.W. Virgin (University of Washington, MO, US). Noroviruses were propagated on RAW 264.7 mouse leukemic monocyte macrophages (ATCC® TIB-71™ as described before (Wobus *et al.*, 2004). Two days after infection, the cells and their culture medium were collected, frozen in liquid nitrogen and thawed three times, and then centrifuged (10 minutes at 1000 *g* then 30 minutes at 5900 *g*). The supernatant was transferred to Amicon® Ultra-15 tangential filters (Merck Millipore LTD, Ireland and centrifuged for 40 min at 1000 *g*. The pellet was resuspended in 1 mL phosphate-buffered saline (PBS) to obtain approximately $10^{10.1}$ copies mL^{-1} corresponding to 10^9 plaque-forming-units (pfu mL^{-1}), or about 8% infectious virus. The concentrated viral suspension was frozen in liquid nitrogen and stored at –80°C, later aliquoted in 10 µl ready-to-use aliquots, refrozen in liquid nitrogen and stored again at -80°C until experiments began. For each column experiment, one 10 µl aliquot was thawed at room temperature, directly diluted with 40 mL of artificial soil solution and homogenized for 30 s. Viruses were quantified by RT-qPCR and plaque assay. For RT-qPCR, 60 µl of viral RNA was extracted from 140 µl of sample following the manufacturer instructions (QIAamp® Viral RNA kit (Qiagen)) and immediately purified with One-Step™ PCR Inhibitor Removal (Zymo Research Corp, CA) according to manufacturer's instructions. RT-qPCR was performed as previously described (Belliot *et al.*, 2008) using a Mx3005P® qPCR machine (Agilent Technologies, France). Plaque assays were performed in 10-fold dilutions of virus inoculum as described before (Wobus *et al.*, 2004).

The 0-15 cm surface layer of calcaric phaeozem (FAO classification) was sampled in the French Limagne Noire, a small region near Clermont-Ferrand (GPS 45.85874 N, 3.20088 E). Average annual rainfall was 566 mm for the period 2010-2016 at the nearest climatic station that is 9 km southwest. The field where sampling took place has been irrigated yearly with treated wastewater from the wastewater treatment plant of *Clermont-Auvergne-Métropole* after an additional lagooning tertiary treatment. It had been cultivated with sugar beet since 2015 and the soil was bare, recently tilled but unsown at sampling on 5 January 2016. The soil was then stored until use at room temperature at a residual moisture of 6.9% dry weight basis. The properties of the 0–15 cm layer were: 3 g kg^{-1} $CaCO_3$, and after decarbonation, 559 g kg^{-1} clay, 222 g kg^{-1} silt, 216 g kg^{-1} sand, 16 5 g kg^{-1} organic C, 1.58 g kg^{-1} total N, 12.7 mg kg^{-1} N-NH_4^+, 29.9 mg kg^{-1} N-NO_3^-. Soil pH(water) was 8.16. Its cation exchange capacity (NF ISO 23470) was 0.465 mol_c kg^{-1} soil, and the concentrations of exchangeable Ca^{2+}, Mg^{2+}, Na^+, K^+, Fe, Mn and Al extracted by cobaltihexamine (NF ISO 23470) were 0.364, 0.0625, 0.00625, 0.011, 0.000386, 0.00013 and 0.0014 mol_c kg^{-1} soil, respectively. Interstratified clays (illite-smectite and illite-smectite-chlorite) were the dominant clays ; their behaviour was affected by complex with organic matter (Bornand *et al.*,

1984). The soil probably contained between 4.5 and 6.1% iron (Bornand *et al.*, 1984) ; 3–4 mm aggregate bulk density was 2.0 g mL^{-1}.

Artificial soil solution was prepared initially from a mixture of ultrapure water and the soil sampled one year later (5:1 w/v) mixed for 30 minutes, and centrifuged at 10 000 g for 30 minutes to recover supernatant which was filtered through NanoCeram® filters. Soil solution was then autoclaved, aliquoted in 40 mL tubes and stored at 4°C until use. Just before experiment, some of the 40 mL aliquots of artificial solutions were optionally enriched with the equivalent of 10 mM $MgCl_2$ or 1.25 mg of fulvic acids (1R105F Nordic Aquatic Fulvic Acid Reference, IHSS). The properties of the artificial soil solution, as well as those for groundwater and wastewater are reported in **Table III-2**.

Table III-2 : Composition of circulating solutions

	Artificial soil solution	Groundwater	Sterilized wastewater
Organic C / mg l^{-1}	11.1	7.89	38.3
Ca^{2+} / mg l^{-1}	12.4	9.68	30.9
Mg^{2+} / mg l^{-1}	3.26	36.7	21.4
K^{+} / mg l^{-1}	13.8	64.7	25.4
Na^{+} / mg l^{-1}	10.9	58.6	12.5
NH_4^{+} / mg l^{-1}	0.35	0.96	0.40
NO_3^{-} / mg l^{-1}	2.07	0.15	0.53
Total N / mg l^{-1}	2.66	1.96	3.13
Cl^{-} / mg l^{-1}	5.23	53.3	92.0
Bore / mg l^{-1}	1.16	0.619	-
S (of SO_4^{2-}) / mg l^{-1}	6.61	23.2	21.8
pH	8.98	8.76	8.60

b. Experimental design and procedure

The experimental design combined experiments on immobilization for batch (soil dispersed in stirred solutions and columns of soil aggregates), as well as experiments on the possibility to remobilize viruses previously removed from the solution (**Figure III.1**). Conditions that were varied between the experiments were the initial column saturation procedure (i.e. under partial vacuum or at atmospheric air pressure), saturation of the column

just before or well before contamination of the circulating solution, temperature, soil moisture, and chemical conditions.

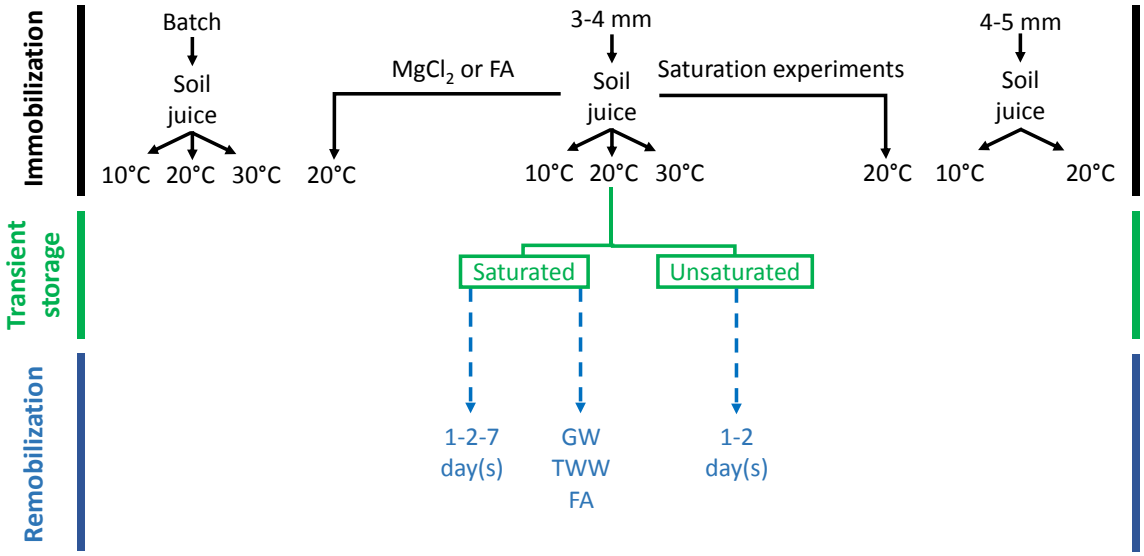

Figure III.1 : Experimental design of the study. GW: groundwater, TWW: treated wastewater, FA: fulvic acids.

For experiments on virus immobilizations, columns (inner diameter and height 18 mm and 3.6 mm, respectively ; total volume 9.2 mL) were initially filled with about 8 g of initially air-dried (6.9 % moisture (dry weight basis)) 3-4 mm or 4-5 mm aggregates of a calcaric phaeozem (FAO classification) ,resulting in about 2.65 mL of solid, 0.93 mL of intra-aggregate pores and 5.62 mL of inter-aggregate pores, and saturated with one of the three artificial soil solutions under vacuum (0.01 MPa air or air) to limit or favour the trapping of air bubbles. The solutions circulated in closed circuits between the columns and reservoirs initially filled with 40 mL of the same artificial soil solution at a flow rate of 4 mL minute^{-1} using a peristaltic pump (**Figure III.2** and **Photo III.1a-b**). Approximately 2.04 mL of water was contained in the tubes between the soil column and the reservoir. The contamination (at time $t = 0$) followed the initial saturation of the column by about 15 minutes or 17 hours depending on whether the quantity of suspended soil colloids, expected to promote transient virus transfer, was to be maximized or minimized. At about 0, 0.25, 0.5, 1, 2, 5, 7 and 24 hours after the contamination, 1.5 mL of soil solutions was sampled in the reservoir, 1 mL of which was filtered at 0.45 µm, the 0.5 mL filtered and unfiltered sampled solutions were distributed in 140 µl, 200 µl and 160 µl for RT-qPCR, cell culture and precautionary storage, respectively.

For virus remobilization, some of the columns previously contaminated with MNV-1 were first stored for different times and moisture conditions and then rinsed by virus-free soil solutions at a flow rate of 4 mL minute^{-1} by creating an open circuit with the reservoir and the column in series (**Figure III.2**). At about 0, 0.17, 0.33, 0.5, 1, 2, 5, 7 hours, 1.5 mL of soil solution was sampled at the column outlet.

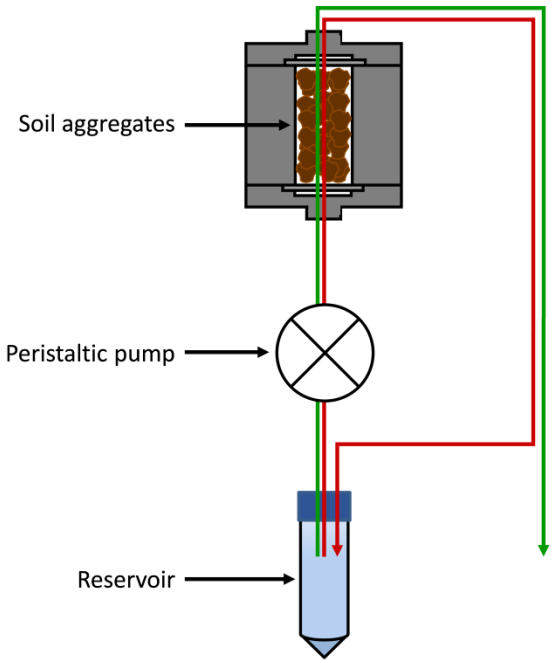

Figure III.2: Experimental setup used for the monitoring of virus immobilisation (flow according to the red path) and virus remobilisation (flow according to the green path.)

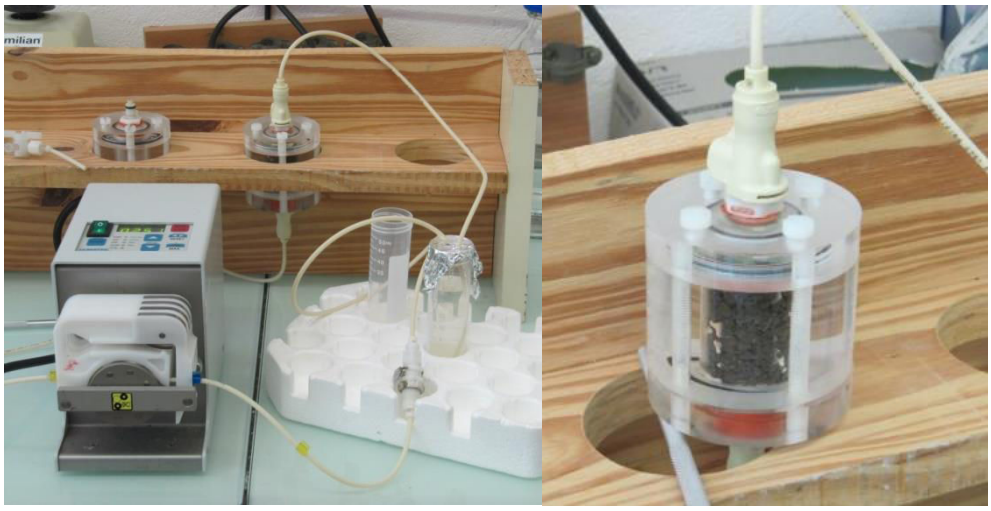

Photo III.1 a-b: (a) Experimental setup used for the monitoring of virus immobilisation, and (b) details of the column filled with soil aggregates.

Batch incubations were performed in sealed 50 mL tubes initially filled with 4 g of air-dried soil and 22.75 mL of the soil solution in order to have the same solution-to-soil ratio as that for column experiments. The tubes were continuously shaken in darkness during 6 hours. They were inoculated 120, 90, 60, 30, 15, 8, 4, 2 and 0 minutes before batch ending with 5 µl of viral suspension to have the same initial contamination per mass of soil as in column experiments. One tube without soil was inoculated and immediately centrifuged to obtain a

zero time point. The tubes were then centrifuged à 10 000 g for 30 minutes, and the supernatant was aliquoted as described above.

At the end of the immobilization experiments and after centrifuging the tubes in the batch experiments, the soil solutions were sampled for subsequent analyses at the Laboratoire d'Analyses des Sols (Arras, France: pH, N-NH_4^+ N-NO_3^-, (spectrophotometric method), total-N (combustion), Ca^{2+}, Mg^{2+}, K^+, Na^+, S (assuming S is only present as SO_4^{2-}), B (ICP-AES or AAF), total and organic C, and Cl^-, NO_3^-, SO_4^{2-} (ionic chromatography).

c. Process modeling, uncertainty analysis and experimental data analysis

For experiments on virus immobilizations with water flowing in a closed loop between the reservoir and the column of soil aggregates, variations in virus concentration in the reservoir may be written:

$$\frac{\partial(C_{\mathrm{r}}+C_{\mathrm{r-c}})}{\partial t} = \frac{Q}{V_{\mathrm{r}}} \times \left((C_{\mathrm{c-m}} + C_{\mathrm{c-m-c}}) - (C_{\mathrm{r}} + C_{\mathrm{r-c}})\right) \quad (1)$$

where t is the time (s), Q the water flow (mL s^{-1}), V_r the volume of water in the reservoir (mL, C_r and $C_{c\text{-}m}$ the concentrations of free viruses (copies mL^{-1}) in the reservoir and in the mobile water between aggregates of the soil aggregate column, respectively, and $C_{r\text{-}c}$ and $C_{c\text{-}m\text{-}c}$ are the concentrations of viruses adsorbed on suspended colloids (copies mL^{-1}) in the reservoir and in the mobile water, respectively.

Virus concentration in the mobile water depends simultaneously on exchange with the reservoir, on exchange of free viruses with intra-aggregate immobile water due to Brownian diffusion of viruses, and on possible adsorption of free viruses at the aggregate outer surface. Considering that we can neglect colloid immobilization on aggregate outer surface, we assume that only free viruses can diffuse within the aggregates:

$$\frac{\partial(C_{\mathrm{c-m}}+C_{\mathrm{c-m-c}})}{\partial t} = \frac{Q}{V_{\mathrm{c-m}}} \times \left((C_{\mathrm{r}} + C_{\mathrm{r-c}}) - (C_{\mathrm{c-m}} + C_{\mathrm{c-m-c}})\right)$$

$$-\left(\left(\alpha_{\mathrm{e}} \times \frac{1-\varepsilon_{\mathrm{m}}}{\varepsilon_{\mathrm{m}}} \times (C_{\mathrm{c-m}} - C_{\mathrm{c-i}})\right) + \left(S_{\mathrm{a}} \times \frac{\partial S_{\mathrm{surf-r}}}{\partial t}\right)\right) \quad (2)$$

where $V_{c\text{-}m}$ is the volume of mobile water between soil aggregates (mL), $C_{c\text{-}i}$ the virus concentration in the immobile water within aggregates (copies mL^{-1}), α_e a coefficient for virus exchange between mobile and immobile waters (s^{-1}), ε_m the inter-aggregate porosity, S_a the aggregate surface (cm^2 per unit of inter-aggregate porosity), and $S_{surf\text{-}r}$ the surface concentration of viruses reversibly immobilized on the surface of the aggregates (copies cm^{-2}). For spherical aggregates, α_e may be approximated by the following equation (Sardin *et al.*, 1991):

$$\alpha_{\mathrm{e}} \approx 15 \times \frac{D_{\mathrm{v}}}{{r_{\mathrm{a}}}^2} \tag{3}$$

where r_a is the aggregate radius (cm), and D_v the virus Brownian diffusion coefficient ($cm^2\ s^{-1}$).

We initially distinguished kinetic reversible and irreversible immobilizations of free viruses from the immobile water, as proposed by Tufenkji (2007):

$$\frac{\partial C_{\mathrm{c-i}}}{\partial t} = \frac{\alpha_{\mathrm{e}}}{\varepsilon_{\mathrm{a}}} \times (C_{\mathrm{c-m}} - C_{\mathrm{c-i}}) - (k_{\mathrm{a-i}} + k_{\mathrm{a-r}}) \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}} \times C_{\mathrm{c-i}} + k_{\mathrm{d-r}} \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}} \times S_{\mathrm{r}} \tag{4}$$

where ε_a is the intra-aggregate porosity, $\rho_{b\text{-}a}$ the bulk density of aggregates ($g\ mL^{-1}$), S_r the concentration of reversibly adsorbed viruses (copies g^{-1}), $k_{a\text{-}r}$ and $k_{a\text{-}i}$ the adsorption coefficients for reversible and irreversible virus adhesion on soil solids, respectively ($mL\ g^{-1}\ s^{-1}$), and $k_{d\text{-}r}$ the desorption coefficient rate for reversible adsorbed viruses (s^{-1}). We consider both reversible and irreversible immobilized viruses S_r and $S_{,}$ respectively (copies g^{-1}), satisfying:

$$\frac{\partial S_{\mathrm{r}}}{\partial t} = k_{\mathrm{a-r}} \times C_{\mathrm{c-i}} - k_{\mathrm{d-r}} \times S_{\mathrm{r}} \tag{5}$$

$$\frac{\partial S_{\mathrm{i}}}{\partial t} = k_{\mathrm{a-i}} \times C_{\mathrm{c-i}} \tag{6}$$

For high $k_{a\text{-}r}$ and $k_{d\text{-}r}$ values and for a constant $k_{a\text{-}r}$-to-$k_{d\text{-}r}$ ratio, $C_{c\text{-}i}$ and S_r satisfy the following equilibrium:

$$\frac{S_r}{C_{c-i}} = \frac{k_{a-r}}{k_{d-r}} = k_D \tag{7}$$

where k_D is the partition coefficient ($mL\ g^{-1}$). Equation (4) may then be replaced by the following equation:

$$\frac{\partial C_{\mathrm{c-i}}}{\partial t} = \frac{1}{\left(1 + k_{\mathrm{D}} \times \frac{m_{\mathrm{s}}}{V_{\mathrm{c-i}}}\right)} \times \left\{\frac{\alpha_{\mathrm{e}}}{\varepsilon_{\mathrm{a}}} \times (C_{\mathrm{c-m}} - C_{\mathrm{c-i}}) - k_{\mathrm{a-i}} \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}} \times C_{\mathrm{c-i}}\right\} \tag{8}$$

where $\left(1 + k_D \times \frac{m_s}{V_{c-i}}\right)$ is often called the retardation factor (Sardin *et al.*, 1991).

Because Equation (8) requires that adsorbed virus concentration S_r is proportional to solution virus concentration $C_{c\text{-}i}$ (not valid if adsorption sites differ from each other and/or if the adsorption cannot exceed a maximum), other mathematical formalizations have been proposed, including the Freundlich isotherms:

$$S_{\mathrm{r}} = k_{\mathrm{F}} \times {C_{\mathrm{c-i}}}^{\frac{1}{n_{\mathrm{F}}}} \tag{9}$$

where n_F is a parameter that indirectly describes the deviation from the proportional model involving partition coefficient k_D, and k_F, a proportionality parameter. Virus concentration changes in the immobile water then satisfy the following equation:

$$\frac{\partial C_{c-i}}{\partial t} = \frac{1}{\left(1+\left(\frac{k_F}{n_F \times C_{c-i}^{\frac{n_F-1}{n_F}}}\right)\times\frac{m_s}{V_{c-i}}\right)} \times \left\{\frac{\alpha_e}{\varepsilon_a} \times (C_{c-m} - C_{c-i}) - k_{a-i} \times \frac{\rho_{b-c}}{\varepsilon_c} \times C_{c-i}\right\} \quad (10)$$

For experiments dedicated to virus re-mobilizations with a flow of water in open circuit between a large reservoir and the column of soil aggregates, we have:

$$C_r = 0 \quad (11)$$

Equations (2)–(10) may then be used with no other changes. However, the experimentally measured quantity is the concentration of viruses in the water at the outlet of the column.

As immobilization experiments over a one-day period showed no significant trends due to the low adsorption properties of the soil and the high uncertainties of molecular quantifications, except for artificial soil solution enriched with $MgCl_2$, most of the parameter estimations came from the remobilization experiments. ε_m and ε_a were estimated assuming a solid density of soil particles and a bulk density of soil aggregates of 2.7 and 2.0 g cm^{-3}, respectively.

As experimental results showed that viruses adsorbed on suspended colloids could be neglected relative to free suspended viruses except when $MgCl_2$ was added to the solution in the reservoir, $C_{r\text{-}c}$, $C_{c\text{-}m\text{-}c}$ and $S_{surf\text{-}r}$ were neglected in the corresponding experiments. A single value of the coefficient of exchange α_e was empirically estimated for all from 3–4 mm remobilization experiments under saturated conditions to ensure no apparent discrepancy between experiments and simulations in the time delimiting the transition between initially sharp and later gentle decrease in virus concentration at the column outlet. A single initial concentration $C_r(t = 0)$ and a single set of parameters k_F, n_F, and k_i were then simultaneously estimated by fitting simulations to experimental data for experiments on immobilizations and for experiments on virus remobilizations for 3–4 mm aggregates at 20°C exposed to artificial soil solution without enrichment in $MgCl_2$ or fulvic acids. By contrast, $C_{r\text{-}c}$, $C_{c\text{-}m\text{-}c}$ and $S_{surf\text{-}r}$ were estimated for experiments with $MgCl_2$ enrichment; we then assumed that $C_{r\text{-}c}$ and $C_{c\text{-}m\text{-}c}$ could be described by partition coefficients, while $S_{surf\text{-}r}$ kinetically varied due to micrometer scale transfer:

$$C_{r-c} = k_D \times \frac{m_{r-c}}{V_r} \times C_r = k_1 \times C_r \quad (12)$$

$$C_{c-m-c} = k_D \times \frac{m_{c-c}}{V_{c-m}} \times C_{c-m} = k_2 \times C_{c-m} \quad (13)$$

with $k_1 = k_2$

$$\frac{\partial S_{\text{surf-r}}}{\partial t} = k_{\text{c}} \times \left(\left(k_{\text{D}} \times \frac{S_{\text{a}}}{V_{\text{c-m}}} \times C_{\text{c-m}} \right) - S_{\text{surf-r}} \right) = k_{\text{c}} \times \left(\left(k_3 \times C_{\text{c-m}} \right) - S_{\text{surf-r}} \right) \quad (14)$$

where k_1, k_2 and k_3 are equilibrium constants and k_c a kinetic constant. As not significant difference was observed for remobilization after the partial drying of soil aggregates, the model was not used specifically for these experiments. For experiments on immobilization after the addition of $MgCl_2$, parameters k_1, k_2 and k_3 were assumed to vary with Mg^{2+} concentration. Finally, the model was fitted to remobilization experiments when groundwater and autoclaved wastewater moved through the column.

4. Results and Discussion

a. Impact of temperature, aggregate size, soil saturation procedure and water filtration on virus concentrations

The means (and standard deviations) of the difference between decimal logarithms of virus concentrations between 0.45 µm-filtered and unfiltered samples were 0.10 (± 0.16), −0.34 (± 0.20) and 0.05 (± 0.25) $\log_{10}$ copies mL^{-1} for soil columns in virus immobilization experiments and saturated with artificial soil solution 12 hours before contamination, soil columns saturated with artificial soil solution 15 minutes before contamination, and artificial soil solution enriched in fulvic acid, respectively.. This suggested that virus adsorbed on colloids dispersed in the moving solution could be neglected with regard to free viruses. By contrast, the addition of $MgCl_2$ led to a mean difference of 0.71 (±0.90) $\log_{10}$ copies mL^{-1} between filtered and unfiltered soil solutions.

The higher virus concentrations in the column saturated under vacuum conditions compared with the one saturated under atmospheric air pressure suggest that viruses were partly immobilized at the air-water interfaces in the column saturated in air; the mean difference of 0.33 (±0.17) $\log_{10}$ copies mL^{-1} suggests that half of the viruses present in solution for the column saturated under partial vacuum condition were immobilized at these interfaces for columns exposed to air during saturation.

Experimental conditions gave negligible virus immobilization, and so did not allow aggregate size effect be highlighted by comparing results for batch, 3–4 mm aggregate column, and 4–5 mm aggregate column. In addition, the coefficient for virus exchange α_e estimated by fitting simulations to experimental data for virus immobilization over 1 week and virus subsequent remobilizations over 7 hours suggest that real aggregate sizes were about one tenth their initial sizes. This agrees with the poor soil structural stability during its wetting, well known for phaeozems (Bornand *et al.*, 1984) and probably results from the covering of clay particles by humic organic compounds (Bornand *et al.*, 1984; Jongmans *et al.*, 1991).

The impact of a higher temperature of 30°C was also assessed but without significant effect (data not shown).

b. Reversible and irreversible immobilization in saturated conditions over a week

For batches and column experiments on virus immobilization, we observe no decrease in virus concentrations over the first 24 hours when the mobile solution was artificial soil solution without enrichment in $MgCl_2$ or fulvic acids (data not shown). The reductions in virus concentrations had to be too small compared to the uncertainty of molecular quantifications and possible heterogeneities of virus concentrations in the solution.

By contrast, column experiments over 1 week led to a reduction of about 3 $\log_{10}$ in virus concentrations (**Figure III.3a**). The viruses that could be remobilised later with artificial soil solution represented a small fraction of the viruses remaining in the soil column after sampling and removal of the reservoir (**Figure III.3**b). The quantities of viruses eluted with beef extract from soil columns after remobilizations were barely equal to the quantities of reversibly immobilised viruses that should have been recovered beyond the 7 hours of remobilization (**Figure III.4**). Non-remobilised viruses may have been irreversibly adsorbed and/or destroyed.

Remobilizations for the samples first subjected to 1, 2, 3 and 7 days of immobilization could be divided into two periods (**Figure III.3**): (i) the first half-hour during which virus concentrations at the outlet of the columns dropped rapidly in a non-exponential time course, and (ii) the period beyond that, during which virus concentrations decreased slowly in an approximately exponential pattern:

$$C_{\mathrm{c-o}}(t) \approx c_1 \times 10^{-c_2 \times t} \quad \text{for} \quad t \geq 0.5\ h \tag{15}$$

where c_1 and c_2 are constants specific to each remobilization experiment. c_1 and c_2 were estimated from linear regressions between $\log_{10}(C_{\mathrm{c-o}}(\mathrm{t}))$ and t, and the quantities of viruses that should have been released after 0.5 hour $\hat{Q}_r(t \geq 0.5)$ were approximated by the equation:

$$\widehat{Q}_{\mathrm{r}}(t \geq 0.5) = \frac{1}{\ln(10) \times c_2} \times \frac{Q}{m_{\mathrm{s}}} \times c_1 \times 10^{-c_2 \times 0.5} \tag{16}$$

$\hat{Q}_{\mathrm{r}}(t \geq 0.5)$ varied with the final $C_{\mathrm{r}}(t)$ before the beginning of remobilization (**Figure III.5**) according to the following equation:

$$\widehat{Q}_{\mathrm{r}}(t \geq 0.5) = 127.6 \times \left(C_{\mathrm{r}}(t)\right)^{\frac{1}{1.6}} \tag{17}$$

Extending Equation (15) to the first half-hour (*i.e.* $\frac{1}{c_2} \times \frac{Q}{m_{\mathrm{s}}} \times c_1$) would have meant multiplying the calculated quantities by 1.08 to 1.38 (results not shown).

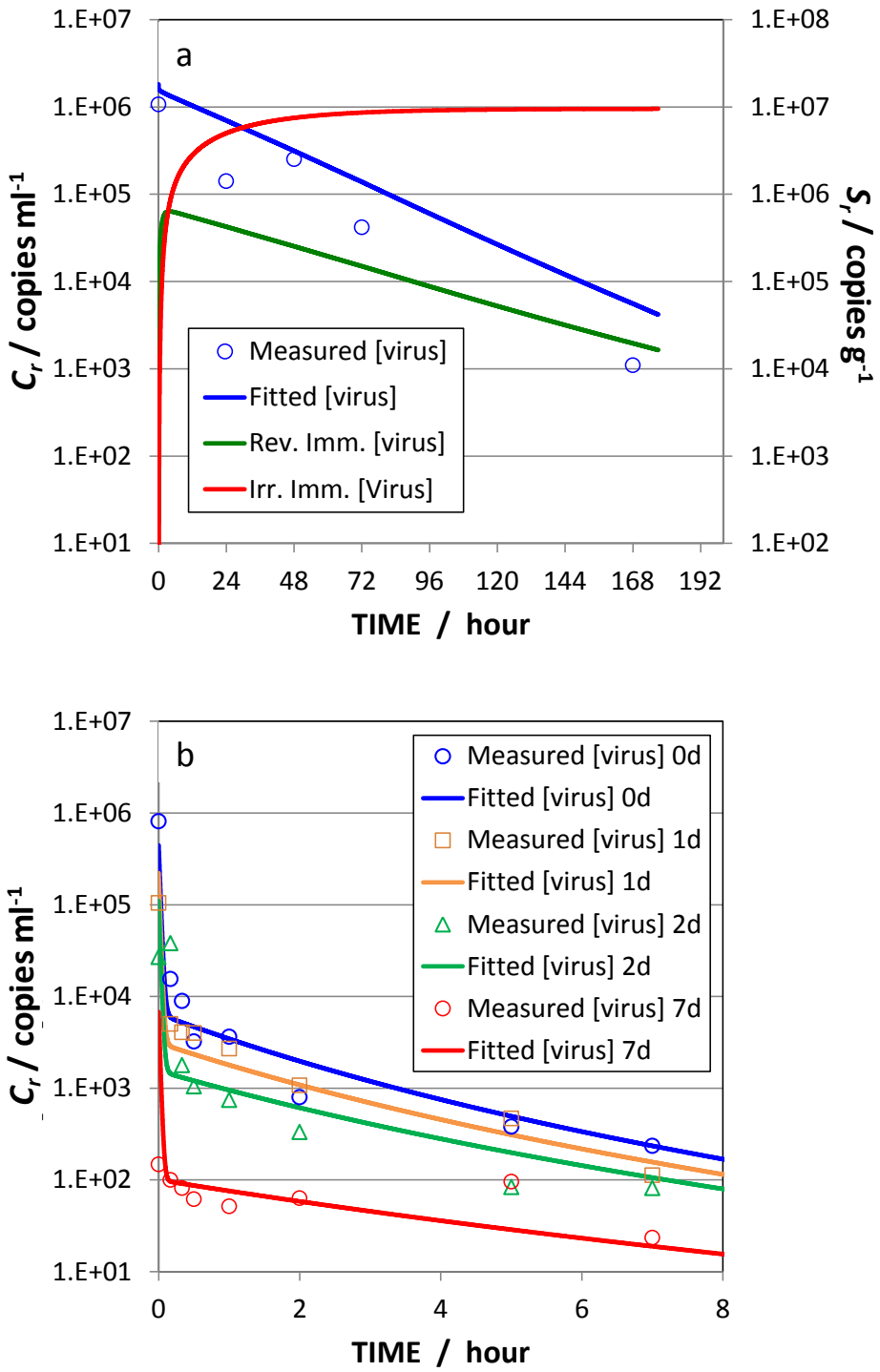

Figure III.3 a-b: Experimental data, and simulations $C_r(t = 0) = 1.82 \times 10^6$ copies.mL^{-1}, irreversible virus immobilization with $k_i = 1.23 \times 10^{-4}$ mL.g^{-1} s^{-1} and reversible immobilization satisfying Freundlich isotherm with $k_F = 1120$ and $n_F = 1.53$ for S_r in copies.g^{-1}. (a) one-week virus immobilization for artificial soil solution as circulating solution; (b) 7 hours remobilizations after 1, 2, 3 and 7-day immobilizations.

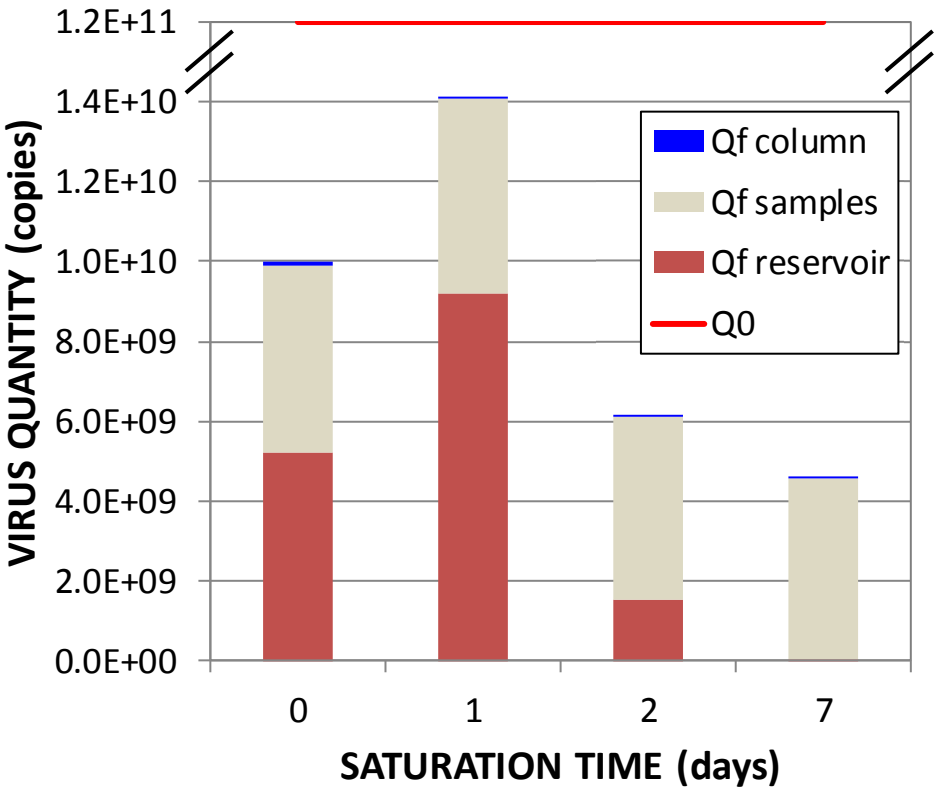

Figure III.4: Quantities of viruses recovered from the sampled soil solution (Qf samples), the reservoir (Qf reservoir) and the soil aggregate columns (Qf column), compared with initial virus quantity contaminating the reservoir (Q0).

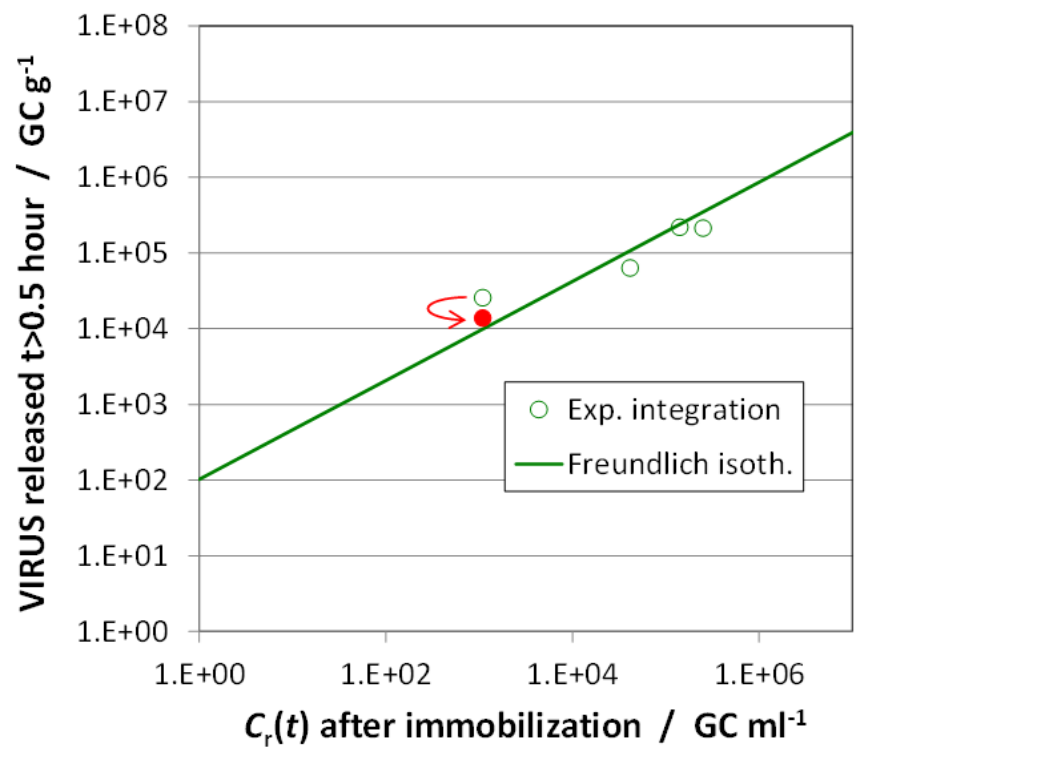

Figure III.5: Estimated quantities of viruses released at the outlet of soil columns after the first half-hour of remobilization experiments with artificial soil solution, as a function of the final virus concentration during preliminary immobilization. Red dot shows the impact of removing one outlier value not taken into account to characterize the Freundlich isotherm.

The lack of proportionality between $\hat{Q}_r(t \geq 0.5)$ and $C_r(t = 0)$ suggests that that immobilization could be described using Freundlich isotherms (Equation (9)), rather than using models based on a partition distribution coefficient k_D (Equation (7)) or kinetic immobilization and remobilization according to Equations (4) and (5a). We described the reversible immobilization of viruses using with $n_F = 1.53$ and $k_F = 102.2$ to fit simultaneously virus immobilization to remobilization experiments (**Figure III.3a, b**). Differences between n_F and c, and k_F and c_1 probably result partly from adsorbed viruses being released during the first half-hour in remobilization experiments.

Regarding the value of k_i=1.23 10^{-4} mL g^{-1} s^{-1}, it appears that our soil is able to definitively immobilize or destroy 90% of the viruses in solution in approximatively 56 hours in our experimental conditions. Moreover, for the estimated k_F and n_F values, the soil could retain approximatively $10^{+2.66}$ copies g^{-1} soil and $10^{+5.93}$ copies g^{-1}, for virus concentration of 10^{+1} copies mL^{-1} and 10^{+6} copies mL^{-1}, respectively.

For our phaeozem containing nearly 60% clay and exposed to a concentration of 10^{+4} copies mL^{-1} of free viruses in solution, the estimated concentration of reversibly immobilized viruses S_r ($4 \times 10^{+4}$ copies g^{-1}) was median with regard to values estimated for various other real soils containing between 5 and 35% clay (Tesson & Renault, 2017) ; for these last soils, minimum and maximum adsorbed concentrations were approximately

between one hundredth and one hundred times more our estimate (Tesson & Renault, 2017). Although virus adsorption may increase with the cation exchange capacity of soils (Lipson & Stotzky, 1983), other factors affect it, including the nature and quantities of cations in solution and the presence of metal oxides (Cao *et al.*, 2010; Syngouna & Chrysikopoulos, 2010). A 0.38 log10 irreversible daily removal of viruses was estimated for the phaeozem. It may have resulted from irreversible immobilization but the fact that no virus was recovered after an attempt to elute them from the soil with beef extract suggests their destruction. This process has most often been ignored in immobilization studies, suggesting that it may be neglected at time scales of immobilization experiments (7–20 hours) (Tesson & Renault, 2017). However, some data on irreversible removal rates have been published: the immobilization rates ranged between 0.001 and 864 day^{-1}.

c. Soil drying impact on the reversibility of immobilizations

After 24 hours of virus immobilization in column experiments saturated with artificial soil solution as moving solution and partial drying to soil moisture (ω) of 0.17 g g^{-1}, 0.24 g g^{-1} and 0.36 g g^{-1} following 1–2 days of desiccation, the columns were gently saturated again with virus-free artificial soil solution to prevent the break-up of soil aggregates. Remobilization experiments were then carried out with virus-free soil solution (**Figure III.6**). Results suggested an effect of desiccation on virus immobilization but further investigations revealed that the observed concentration difference could also be observed from the initial contamination of columns leading to the conclusion that it may be due to lower virus concentration in some aliquots.

The final state of viruses at the end of the desiccation periods closely depends on the final soil moisture and aggregate bulk density. With an aggregate bulk density $\rho_{b\text{-}a}$ of about 2.0 g mL^{-1} for this soil, the aggregates were never desaturated, even for the lowest of the experimental soil moisture (ω)=0.17 g g^{-1} dry weight basis. This could explain the lack of a drying effect, as water suction probably remained lower than about 2 MPa, ensuring that all the pores of diameter greater than 150 nm remained water-saturated (Fiès, 1992; Renault, 1988).

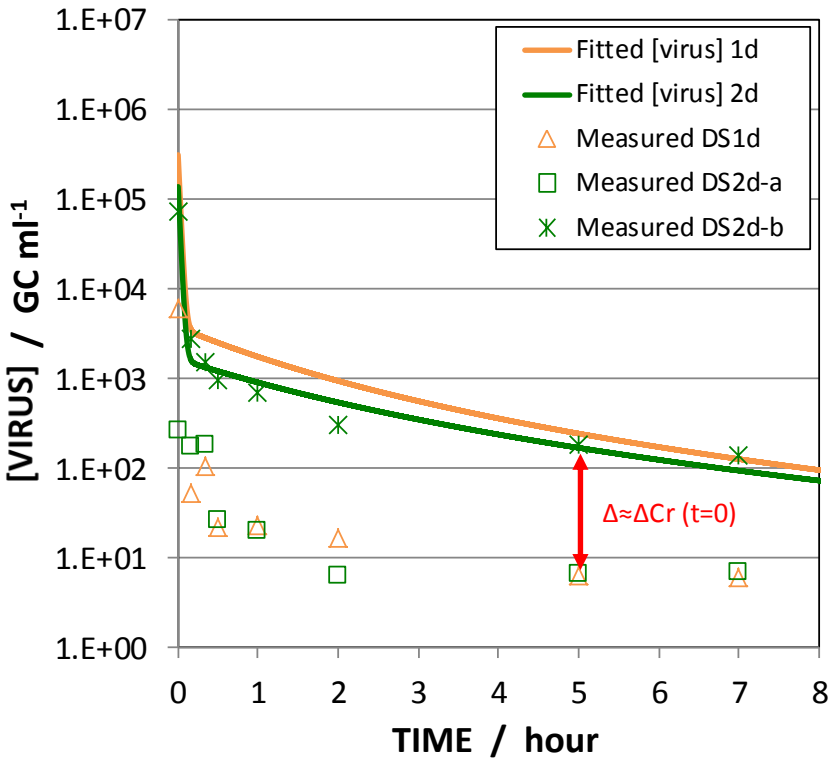

Figure III.6: Experimental data for 7 hours virus remobilization experiments after a partial desaturation of the soil columns for 1 day (column DS1d) with final $\omega = 0.36$ g g^{-1} or 2 days (column DS2d-a) with final $\omega = 0.24$ g g^{-1} and column DS2d-b with final $\omega = 0.17$ g g^{-1}. (Simulations were performed using $C_r(t = 0)$, k_i, k_F and n_F estimated for immobilization and remobilization in columns always saturated (see values in caption of **Figure 3a, b.**))

d. The impact of geochemistry on immobilization and subsequent desorption

Virus immobilization was assessed according to several different geochemical characteristics of the circulating solution, with artificial soil solution alone or enriched by either 10 mM $MgCl_2$ or 1.25 mg of fulvic acids. While no significant effect of adding fulvic acids was observed, the addition of $MgCl_2$ led to a important immobilization of viral particles, with a rapid decrease in about 3 hours of the concentrations of viruses in unfiltered samples of more than 2 $\log_{10}$ copies mL^{-1} and concentrations of viruses in filtered samples being approximately one tenth of the concentrations in unfiltered samples, except at the last time (**Figure III.7**), i.e. approximately 90% of viruses in the circulating solution were adsorbed on dispersed colloids, and 10% were free. After the first 3 hours of immobilization, the virus concentrations increased and tended towards values probably equal to about one-tenth of the initial concentration, probably with a narrowing of the differences between filtered and

unfiltered samples; unfortunately, we could not determine whether the final concentrations of filtered and unfiltered samples were equal. As partial exchange of Mg^{2+} in solution with cations retained on soil cation exchange sites (mainly Ca^{2+}) led to 3.1 mM Mg^{2+} final concentration, we assumed that $[Mg^{2+}]$ varied according to the following equations:

$$[\mathrm{Mg}^{2+}](t) = \left([\mathrm{Mg}^{2+}](t=0) - [\mathrm{Mg}^{2+}](t=\infty)\right) \times e^{-0.1\times t} + [\mathrm{Mg}^{2+}](t=\infty) \quad (18)$$

We assumed the following effects of $[Mg^{2+}]$ on constants k_1, k_2 and k_3:

$$k_1(t) = k_1(t=0) \times 10^{1-\frac{[\mathrm{Mg}^{2+}](t=0)}{[\mathrm{Mg}^{2+}](t)}} \quad (19)$$

$$k_2(t) = k_1(t) \quad (20)$$

$$k_3(t) = k_3(t=0) \times 10^{1-\frac{[\mathrm{Mg}^{2+}](t=0)}{[\mathrm{Mg}^{2+}](t)}} \quad (21)$$

with k_1 $(t = 0)$=k_2 $(t = 0)$=10, and k_3 $(t = 0)$=6 10^4. In addition k_c was set to 5 10^{-5} s^{-1}. In doing so, we obtained simulated values close to experimental ones during the first 3 hours (**Figure III.7**); beyond this time, the re-increase in free virus concentrations was approximately simulated, while the re-increase in the concentration of viruses adsorbed on dispersed colloids was greatly underestimated. This last difference probably resulted from simultaneous variations in dispersed colloid concentrations that are not described in the model. Moreover, k_F value did not affect virus immobilization in those conditions, suggesting that virus immobilization within soil aggregates could be neglected, compared to virus immobilization on aggregate outer surface and dispersed colloids.

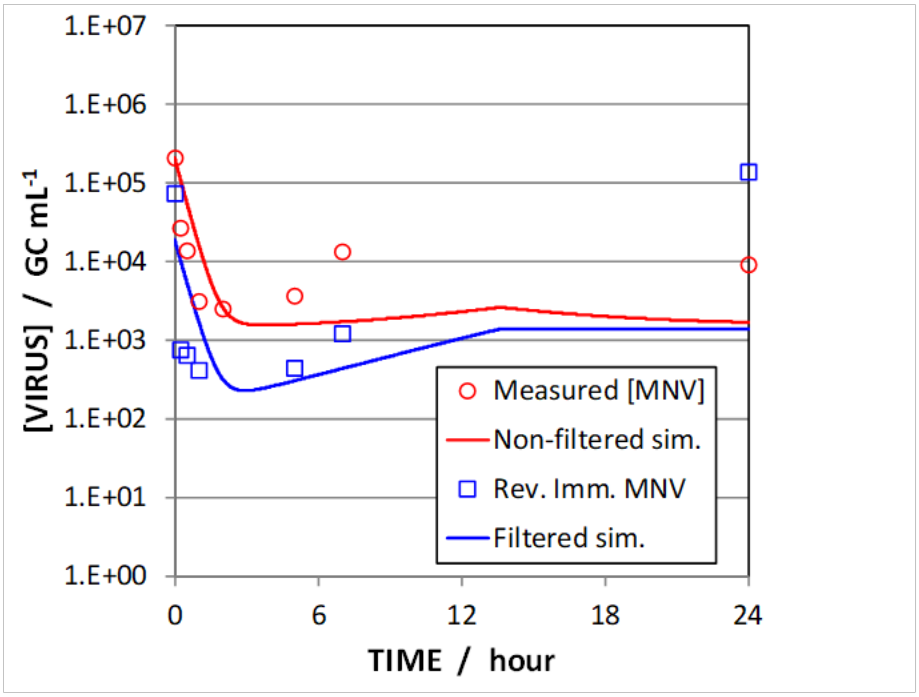

Figure III.7: Experimental data for 24 hours virus immobilization experiments for circulating artificial soil solution initially enriched with 10 mM of $MgCl_2$. (Simulations were

performed without fitting to experimental data, assuming a partial decrease in [Mg^{2+}]) due to exchange with other cations retained on cation exchange sites of clays and organics

Remobilization experiments were also conducted on aggregate soil columns previously exposed to 24 hours of contamination at 20°C and contaminated artificial soil solution.Taking into account the differences between columns for remobilizations with an artificial soil solution, and with either groundwater (−1.3 $\log_{10}$ copies mL^{-1}) or autoclaved wastewater (−0.35 $\log_{10}$ copies mL^{-1}), we did not observe any large differences between the column exposed to virus-free artificial solution and the one exposed to autoclaved wastewater (**Figure III.8**) probably because the effects of increasing divalent cation concentrations are counterbalanced by the effects of increasing soluble organic matter content (**Table III-2**). By contrast, remobilization of viruses for contaminated column exposed to groundwater gave lower remobilization resulting from the groundwater geochemical characteristics, especially its higher Mg^{2+} concentration (**Table III-2**).

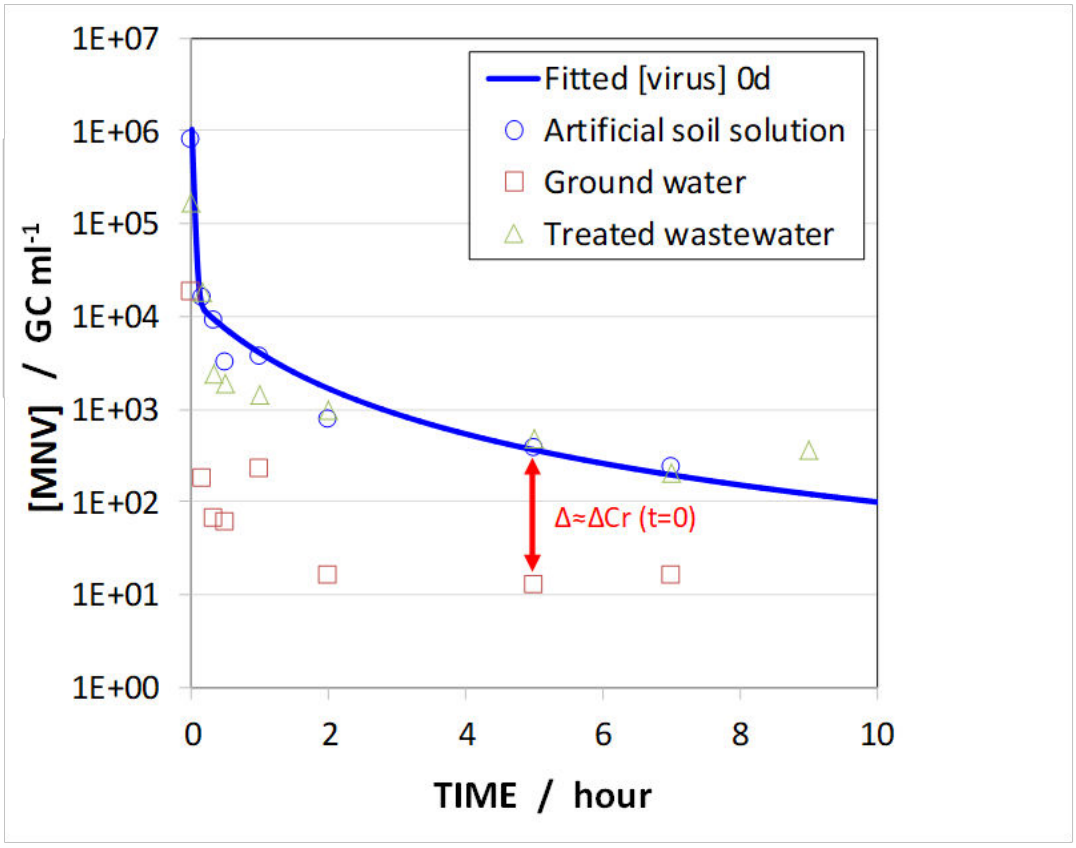

Figure III.8: Experimental virus remobilization experiments for circulating artificial soil solution, autoclaved wastewater, and groundwater, after a first virus immobilization for soil aggregate columns at 20°C and exposed to contaminated artificial soil solution.

5. Conclusions and perspectives

Our aim was to collect new experimental data for a calcaric phaeozem and write a mathematical model to describe reversible virus immobilization and irreversible removal (due to irreversible immobilization and/or virus degradation. To our knowledge, our study is the first one on a real soil containing more than 35% clay (Tesson & Renault, 2017). The clay content and its nature, together with the organic matter content, give this phaeozem a high cation exchange capacity; while this value is often positively correlated to virus immobilization, our phaeozem retained few murine noroviruses. This could have resulted from the complex association between smectite and organic matter as it is well known for Limagne Noire soils (Bornand *et al.*, 1984; Jongmans *et al.*, 1991). However, the high daily virus removal in this phaeozem could have resulted from the simultaneous presence of iron oxides and a solution pH just above neutrality (Zhuang and Jin, 2008 ; Murray & Laband, 1979).

It was possible to simulate virus fate in the phaeozem with a model combining free and colloidal transport of viruses in mobile water, exchange of free viruses between mobile and immobile waters, kinetic virus removal, and virus reversible adsorptions on suspended colloids, aggregate surface and within aggregates. For experiments without the added Mg^{2+}, colloidal transport of virus could be neglected to their low immobilization on soil. By contrast, Mg^{2+} enrichment greatly increased virus and so colloidal transport of viruses had to be taken into account, together with virus adsorption on aggregate outer surfaces, outreaching the simple model of exchange between mobile and immobile water.

Practically, a first approximation of the virus average residence time in this phaeozem could be the soil water holding capacity–to–the daily maximum evapotranspiration ratio is probably higher than 8 days. This duration corresponds to 3 log10 virus removal; however, viruses brought to the soil quickly disperse in the water, especially in its mobile fraction that that may then be easily absorbed by plant roots.

Chapitre IV

Discussion générale – Conclusions

1. Rappel des objectifs et finalités de la thèse :

Le monde doit faire face à des problèmes croissants de ressources en eaux douces, en quantité et en qualité. Ces problèmes résultent notamment de l'accroissement démographique qui va de pair avec la surexploitation de ces ressources et du rejet accru d'eaux usées généralement non ou insuffisamment traitées. La réutilisation des eaux usées présente actuellement de nombreux atouts en ajoutant une nouvelle ressource en eau et en limitant leur rejet dans l'environnement. Elle amène toutefois *a priori* à déplacer des pollutions, dont celles par les virus entériques pathogènes de l'Homme du compartiment "eaux douces" aux compartiments "sol" et "plante", voire "eaux souterraines". Les risques sanitaires réels associés respectivement aux rejets en rivière ou à la réutilisation en irrigation agricole dépendent alors du devenir environnemental des virus entériques pour chacune de ces modalités.

Afin d'aboutir à terme à une évaluation quantitative des risques viraux associés à différents scénarios se distinguant par le mode de gestion de eaux usées (traitements, rejets et/ou recyclage, stockage éventuel en réservoir, type de réutilisation …), la thèse avait pour objectifs de caractériser et modéliser (i) le devenir des virus entériques pour un scénario de rejet en rivière, et (ii) le devenir d'un virus modèle dans le sol ; avec ce deuxième objectif, elle se voulait contribuer à la connaissance plus générale du devenir des virus pour un scénario de réutilisation en irrigation agricole. Cette étude, et notamment l'objectif (ii), a été complétée par un travail sur la contamination de plantes cultivées sur le même sol auquel nous avons participé (cf. Annexe I). En amont, ces objectifs nous ont amenés à dédier une partie de notre travail aux liens possibles entre données épidémiologiques et excrétion globale de virus entériques à l'échelle d'un bassin de collecte des eaux usées.

Dans le cadre de ce travail, nous n'avons pas été jusqu'à la réalisation d'une évaluation quantitative du risque viral (QVRA) adaptée à différents scénarios de gestion des eaux usées ; ces scénarios seraient d'ailleurs à réfléchir avec les différents acteurs potentiels en initiant une démarche de recherche de type participatif.

2. Devenir des virus dans un contexte de rejet : acquis et limites du travail

Les quantifications virales dans les eaux de la région de Clermont-Ferrand ont été effectuées entre juin 2015 et août 2016. Le virus de l'hépatite A n'y a jamais été détecté au cours de cette période. Ceci peut s'expliquer par le fait que ce virus n'est pas endémique en France et que les cas d'hépatite A sont très majoritairement des cas importés par des personnes venant d'Afrique du Nord, d'Asie du Sud-Est ou d'Amérique latine (Jacobsen & Wiersma, 2010).

Tous les autres virus étaient classiquement détectés dans les eaux usées brutes entrant dans la station d'épuration des eaux usées *'Les Trois Rivières'*, avec notamment pour les norovirus GII un pic de concentration observé en hiver, en accord avec la saisonnalité de ce virus (Prevost *et al.*, 2015b). Sa concentration fluctuait autour de $10^{+5,5}$ copies.L^{-1} durant la période épidémique, soit des valeurs proches de celles trouvées dans plusieurs études : de l'ordre de 10^{+6} copies.L^{-1} pour Van den Berg et al. (2005) et de 10^{+7} copies.L^{-1} pour Campos et al. (2016).

Nous avons tenté d'établir un lien entre les concentrations virales dans les eaux usées brutes et l'épidémiologie, en estimant une proportion de gastroentérites aiguës d'étiologie virale et en simulant pour chaque individu infecté, porteur symptomatique ou asymptomatique, une cinétique d'excrétion de virus ; à notre connaissance, cette étude est la première tentative de lien entre données épidémiologiques accessibles et production de virus. Pour permettre la comparaison entre les concentrations virales simulées sur toute l'année sans distinction d'espèces et les données expérimentales accessibles à certaines dates pour différentes espèces, nous avons défini une concentration "expérimentale" en virus équivalents comme étant la somme pondérée des concentrations mesurées. Après ajustement des coefficients de pondération, le modèle reflétait globalement correctement les variations temporelles de charge virale des effluents urbains, notamment la multiplication par un facteur de l'ordre 100 du débit viral durant l'hiver 2015-2016 par rapport aux débits viraux des périodes estivales 2015 et 2016 (cf **Figure II.7**) ; ces variations sont conformes aux variations saisonnières rapportées dans la littérature (Ahmed *et al.*, 2013; Kazama *et al.*, 2015; Prevost *et al.*, 2015b). Les pondérations estimées des concentrations virales mesurées sont un indicateur de la contribution unitaire de chacun de ces virus aux cas estimés de gastroentérites aiguës d'étiologie virale. La contribution unitaire estimée du norovirus GII (coefficient de pondération égal à 10^{-2}) est très élevée par rapport à celle des autres virus (coefficients de pondération inférieurs ou égaux à 10^{-4}), conformément à son rôle prépondérant dans les gastroentérites aiguës dans l'hémisphère nord (Belliot *et al.*, 2014; Kirk *et al.*, 2015; Kowalzik *et al.*, 2015; Pires *et al.*, 2015; Wit *et al.*, 2001), et notamment en France depuis plus d'une dizaine d'années (Bon *et al.*, 2005; van Beek *et al.*, 2013). La non-contribution du norovirus GI et de l'entérovirus à la concentration en virus équivalents indique le poids unitaire mineur de ces pathogènes dans les estimations de gastroentérites aiguës d'étiologie virale, bien que les estimations de leurs coefficients de pondération (zéros) soient certainement entachées de fortes incertitudes.

Les écarts subsistants entre débits viraux simulés et débits "expérimentaux" de virus équivalents arrivant à la station d'épuration peuvent avoir plusieurs origines. Ils renvoient pour partie à des limites qui sont autant de potentialités d'amélioration du modèle d'excrétion et de devenir environnemental des virus entériques :

- les liens entre données épidémiologiques (incluant la consommation de médicaments) et gastroentérites aiguës d'étiologie virale sont au minimum mal évalués pour la classe

d'âge < 5 ans, les antiémétiques étant contre-indiqués dans leur cas (Assathiany *et al.*, 2013; Pfeil *et al.*, 2008) ;

- la faible taille de la population du bassin de collecte des eaux usées (environ 238 000 habitants avec chaque jour de 0 à 198 cas nouveaux de gastroentérites aiguës amenant à consultation médicale) peut générer de grosses incertitudes sur le nombre réel de gastroentérites aiguës d'étiologie virale ;
- l'utilisation d'un seul critère discriminant, i.e. la proportion de vomissement parmi les personnes malades, favorise les incertitudes. L'analyse gagnerait à être enrichie par les deux autres critères les plus discriminants pour les gastroentérites aiguës d'étiologie virale, i.e. les diarrhées muqueuses et la fièvre (Costantini *et al.*, 2016; Van Cauteren *et al.*, 2012). De plus, il semble que notre méthodologie gagnerait à prendre en compte une variation avec l'âge de la proportion de vomissements associée aux gastroentérites aiguës d'étiologie virale (Costantini *et al.*, 2016) ;
- le choix de ne pas tenir compte de la variabilité inter-individus de l'excrétion virale dans les selles en termes de concentrations et de durée d'excrétion, mais aussi en termes de masse de selle, est certes adapté à une démarche de confrontation entre mesures expérimentales et simulations, mais il peut être trop simplificateur (Teunis *et al.*, 2015). La prise en compte explicite de ces variabilités serait au minimum à envisager pour la simulation de scénarios contrastés et la prise en compte d'un certain aléa. La prise en compte des virus dans les vomissures améliorerait probablement les analyses (Kirby *et al.*, 2016) ;
- l'hypothèse d'absence d'abattement viral dans les égouts peut être discutée au vu des connaissances sur l'immobilisation des virus par les biofilms (Heistad *et al.*, 2009), et sur la destruction de virus en présence d'oxydes métalliques (Gotkowitz *et al.*, 2016; Hvitved-Jacobsen *et al.*, 2013) ou de conditions réductrices (Roehrdanz *et al.*, 2017). Cette hypothèse doit être pondérée par le temps de résidence des virus dans ces égouts avant qu'ils n'arrivent à la station (Nielsen *et al.*, 1992).

L'abattement *a priori* élevé des concentrations virales dans la station d'épuration (cf. (Erreur ! Source du renvoi introuvable.) explique la faible fréquence de détection des virus ntériques sur les prélèvements de 1 L faits en sortie de station ; les rejets réels de virus par la station étaient mieux estimés à partir de l'accroissement des débits viraux évalués sur des prélèvements de 100 L dans l'Artière entre l'amont et l'aval du point de rejet de la station.

Les concentrations en virus dans l'Artière en amont des rejets de la station d'épuration étaient de l'ordre de 10^1 à 10^4 copies.L^{-1} pour le norovirus GI ; les autres virus recherchés n'ont été que très peu détectés au cours de l'étude (résultats non montrés). En contraste, les virus étaient la plupart du temps détectés en aval, parfois à des concentrations proches du seuil de détection avec une abondance plus marquées des adénovirus, quasi-systématiquement détectés à des concentrations alors de l'ordre de 10^3 – 10^6 copies.L^{-1}, en accord avec la plupart des études menées sur le sujet (D'Ugo *et al.*, 2016; Prevost *et al.*, 2015b; Vergara *et al.*, 2016;

Wyn-Jones *et al.*, 2011). L'accord relativement bon entre concentrations virales simulées et concentrations "expérimentales" en virus équivalents montre la capacité du modèle à simuler les processus en jeu et, indirectement, la participation des rejets de la station de traitement aux contaminations virales de la rivière. En contraste, nos résultats ne permettent pas d'établir un constat clair sur la conservation des débits de virus sur le tronçon de l'Artière entre le site de prélèvement en aval des rejets de la station et celui juste en amont de la confluence avec l'Allier. Les concentrations bactériennes moins variables que les concentrations virales (**cf. Table II-3**) suggèrent des incertitudes importantes dans les quantifications moléculaires des virus. Les quelques fois où elles sont similaires, les variations des concentrations virales et bactériennes pourraient suggérer l'impact d'autres processus (apports latéraux d'eau ou d'une contamination additionnelle) ; les débits linéaires élevés (de l'ordre de 1 $m.s^{-1}$) sur le tronçon sont peu compatibles avec un abattement viral important. Les résultats obtenus sur l'Allier entre l'amont, enrichi par les arrivées de l'Artière, et l'aval de la confluence, amènent à des conclusions similaires même si, dans ce cas, les conditions de prélèvement peuvent être mises en cause : du fait de la largeur de l'Allier au niveau des points de prélèvement (entre 75 et 80 m) et de l'absence de pont ou de barque pour atteindre le milieu du cours d'eau, nos prélèvements ont été réalisés à moins de 2 m des berges. À cette distance, les eaux sont plus stagnantes et peuvent mal se mélanger avec les eaux plus animées du centre de la rivière.

3. Devenir des virus dans le sol dans un contexte de réutilisation d'eau usée : acquis et limites du travail

L'étude du devenir de virus entériques dans le sol a été menée avec le norovirus murin, souche CW1, et un phaeozem calcaire provenant d'une parcelle de Limagne noire irriguée par des eaux usées. Le choix du virus modèle a été motivé par la proximité de ses propriétés de surface avec celles des norovirus GI et GII, agents étiologiques majeurs des gastroentérites aiguës (point isoélectrique (Bolton *et al.*, 2013), résistance aux pH acides, taille (Wobus *et al.*, 2006)) et par sa facilité de mise en culture à des fins de production, voire de suivi du caractère infectieux. Toutefois, nous n'avons pas de données sur leurs mouillabilités respectives ; les deux types de virus se distinguent par leurs récepteurs cibles (HBGA ou acide sialique (Tan & Jiang, 2010), cette dernière différence étant probablement sans conséquence sur le devenir du virus dans le sol.

Afin d'éviter des artefacts liés à une immobilisation de virus, voire à leur destruction à l'interface "eau –air – polypropylène (des tubes)" mise en évidence pour le bactériophage MS2 par Thompson et al. (1998) ou dans la solution, nous avons suivi sur sept jours à 20°C l'évolution de la concentration en norovirus murin dans 40 mL de solution artificielle de sol déposée dans des tubes de 50 mL. Les prélèvements réalisés à la contamination ainsi que 1, 2, 3 jours après n'ont révélé aucun abattement temporel ; les prélèvements à 7 jours furent les seuls à révéler un abattement, de moins d'un log_{10} dans ce cas. Au vu des ordres de grandeurs

des évolutions observées en présence de sol, cet abattement a été négligé ou considéré comme participant de façon mineure aux évolutions observées.

L'immobilisation réversible et/ou la disparition des virus à 20°C dans des colonnes d'agrégats saturées par une solution artificielle de sol ne pouvaient être mises en évidence que pour des durées supérieures à 24 heures ; on observait alors un abattement progressif de 0,38 $log_{10}.jour^{-1}$ de la concentration en virus dans la fraction mobile de la solution de sol (**cf. Figure III.3**). L'abattement correspondait pour l'essentiel à une immobilisation définitive des particules virales sur les agrégats, voire à la destruction desdites particules que nous n'avons pas réussi à remobiliser par une procédure d'élution utilisant une solution d'extrait de bœuf à pH 9,5 (la méthode pourrait être améliorée en réalisant l'élution sous sonication). Si elle existe, la destruction des virus pourrait résulter de tensions interfaciales (Thompson *et al.*, 1998) éventuellement à la surface d'oxydes, hydroxydes ou oxyhydroxydes métalliques présents (Murray & Laband, 1979; Park & Kim, 2015; Warnes *et al.*, 2015; Zhuang & Jin, 2008), provoquant la désagrégation des protéines de la capside virale et la mise à nu de l'ARN viral extrêmement fragile dans l'environnement. À différentes dates après le début de la contamination initiale, le balayage des colonnes par une solution de sol non contaminée a permis de remobiliser pour partie les virus réversiblement immobilisés ; la quantité de virus réversiblement immobilisée sur le sol pouvait être décrite par une isotherme de Freundlich avec une forte non-linéarité de la relation entre concentration virale en phase solide et concentration virale en solution. Les concentrations virales au cours de l'immobilisation initiale ou des rinçages ultérieurs n'ont pas révélé d'écart de concentration entre solution filtrée à 0,45 µm et solution non filtrée, suggérant que les virus en suspension dans la solution circulante étaient principalement libres, i.e. non adsorbés à la surface de colloïdes dispersés dans cette même solution. Les suivis expérimentaux ont pu être simulés par un modèle prenant en compte les transferts (convection avec l'eau mobile, échange diffusif entre eau mobile et eau immobile des agrégats), l'immobilisation réversible et la disparition irréversible. Les paramètres estimés pour ajuster les simulations aux données expérimentales étaient dans la gamme des données de la littérature (Tesson & Renault, 2017) avec toutefois une immobilisation réversible des virus faible malgré la teneur en argile et la capacité d'échange cationique élevées de notre sol ; la faiblesse de cette immobilisation pourrait résulter des complexes smectite-matière organique spécifiques aux phaeozems qui rapprocheraient les feuillets d'argiles (Bornand *et al.*, 1984) et réduiraient alors les possibilités d'adsorption du virus.

Un ajout de $MgCl_2$ à la solution artificielle de sol en phase de contamination (correspondant à une concentration initiale de 10 mM) a fortement déplacé les équilibres avec une immobilisation rapide et très importante des virus, et 90% des virus en suspension présents sous forme adsorbée sur des colloïdes retenus par des filtres à 0.45 µm. Après quelques heures, la concentration des virus en suspension dans l'eau mobile remonte légèrement, probablement en conséquence d'un échange partiel entre Mg^{2+} initialement en solution et les cations adsorbés sur le complexe d'échange cationique du sol ; la concentration finale en Mg^{2+} après 24 heures n'est plus que de 3.1 mM). Le modèle a été adapté pour

prendre en compte (i) le partage entre virus libres et virus adsorbés sur des colloïdes en suspension dans l'eau mobile, (ii) l'adsorption spécifique sur la surface externe des agrégats pour tenir compte de l'inadéquation de la description simplifiée des échanges entre eaux mobile et immobile, et (iii) très indirectement l'évolution de la concentration en Mg^{2+} sur les paramètres relatifs à l'adsorption des virus. Il permet ainsi de refléter correctement les grandes tendances expérimentales.

Les autres facteurs testés n'ont pas montré d'impact marqué dans notre étude. Notamment, la désaturation de colonnes contaminées de sol n'a pas accru l'irréversibilité des immobilisations, les procédures de désaturation n'ayant pas permis d'aller jusqu'à la désaturation des agrégats constitutifs des colonnes de sol ; le potentiel de l'eau n'était probablement pas à des valeurs telles que les forces en jeu aux interfaces "air-eau-solide" puissent favoriser la désagrégation des protéines constitutives des capsides virales.

Le modèle présente des limites liées aux simplifications qu'il suppose, notamment la concentration virale uniforme dans l'eau mobile en tout début d'expérimentation, et la description des échanges entre eau mobile et eau immobile à partir de concentrations moyennes dans chacun de ces compartiments (aboutissant parfois à la nécessité d'ajouter une description de l'adsorption à la surface des agrégats comme correctif à l'excès de simplification). Au-delà, l'extension de ce modèle ou d'autres modèles à la diversité des contextes réels peut se heurter au minimum à deux types de défis : le passage à des contextes de sols naturels avec par exemple la nécessité de prise en compte d'écoulements préférentiels et des variations temporelles d'ambiance (géo)chimique.

4. Défis à aborder pour atteindre les finalités du travail

Notre thèse portait sur la caractérisation et la modélisation du devenir environnemental de virus entériques (i) pour des scénarios de rejet en rivière, et (ii) pour des scénarios de réutilisation en irrigation agricole en se limitant dans ce deuxième cas au seul transport d'un virus modèle dans le sol. Il s'agit *in fine* d'aboutir à une évaluation quantitative des risques sanitaires d'origine virale pour divers scénarios, afin d'éclairer les décisions en matière de gestion des eaux usées ; d'autres aspects peuvent contribuer à ces décisions comme leurs impacts environnementaux, leurs impacts sur les activités potentiellement utilisatrices d'eaux usées (agriculture …), leurs durabilités économiques et leurs acceptabilités sociales.

Plus spécifiquement sur le volet "devenir environnemental des virus entériques pathogènes de l'Homme", il serait nécessaire d'accroitre les acquis dans différents domaines et d'aboutir à une évaluation quantitative des risques viraux (QRVA) associés aux différentes de la gestion des eaux usées. Les axes qui semblent prioritaires sont :

- la description et la modélisation du devenir des virus dans les réseaux d'assainissement entre leur excrétion et leur arrivée en station, en se concentrant sur l'évolution des eaux usées et des concentrations virales durant leur trajet. Ce travail pourrait notamment prendre en compte le temps de transit des eaux, les conditions biogéochimiques rencontrées et d'autres aspects liés à l'histoire du réseau ;
- la description de l'abattement viral en station d'épuration à l'échelle des différents bassins de traitement des eaux usées en investiguant en profondeur pour chacun d'eux les effets du niveau de contamination virale en entrée de bassin, du temps de séjour des eaux, et des traitements biologiques et physicochimiques visés ;
- la description du devenir des virus au niveau de la plante, avec la persistance de la contamination virale en surface de plante en fonction des rinçages, et après internalisation dans la plante avec une étude poussée de la redistribution des virus au sein du végétal, comme déjà abordé dans une étude à laquelle nous avons contribué (cf. Annexe I). Cette étude prendrait bien évidemment en compte le devenir des virus dans le sol avant qu'ils puissent atteindre les racines et être internalisés à leur niveau ;
- la description des flux de virus arrivant à l'aquifère suite à un cumul d'irrigations par des eaux contaminées.

Ces acquis sur le devenir environnemental des virus entériques pathogènes de l'Homme doivent déboucher sur une évaluation quantitative des risques microbiens (QMRA) personnalisée pour ces risques viraux (QVRA). Elle suppose que l'on puisse renseigner aussi l'inactivation des virus dans l'air, l'eau, le sol et la plante. Elle nécessite par ailleurs au minimum de prendre en compte la dimension stochastique de plusieurs phénomènes : climat, épidémiologie, variabilité entre individus … Dans cette optique-là, nous avons déjà réuni des données épidémiologiques et hydrologique sur la période 2010-2016, afin de pouvoir simuler les excrétions virales (et la variabilité de celles-ci entre individus) et le devenir des virus dans les eaux pour des années climatiques différentes et pour des scénarios de gestion des eaux usées qui pourraient être définis à parti de nos interactions avec les acteurs régionaux de la gestion des eaux.

Dans ce cadre, notre étude constitue déjà un premier élément d'approche qui pourrait être amélioré et complété pour ce qui est du devenir environnemental des virus entériques. Il pourrait en parallèle ou ultérieurement par une évaluation quantitative des risques, mais mener de front ces deux volets peut permettre de mieux cerner les besoins de description sur le devenir des virus entériques pour affiner les modèles là où cela s'avèrerait nécessaire.

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Annexe I

Surface and internalized contaminations of green onions

* Cette Annexe correspond au Chapitre IV du *final report of the Full project n°1403-050* financé par Agropolis Fondation et intitulé *"Wastewater reuse for agricultural irrigation as an alternative to wastewater discharge in river; environmental fate of human enteric viruses present in treated wastewater"*. Il doit donner lieu à la soumission d'un article cosigné par Renault P., Tesson V., Ben Jemaa N., Linguet S., Belliot G., Tomas S.

Agropolis Fondation; final report of the Full project n°1403-050

Wastewater reuse for agricultural irrigation as an alternative to wastewater discharge in river; environmental fate of human enteric viruses present in treated wastewater

Chapter IV. Surface and internalized contaminations of green onions

IV.1. State of the art and objectives

In the US, noroviruses are by far the leading cause of outbreaks associated with leafy vegetable consumption[1], and leafy vegetables cause more norovirus outbreaks than seafood[2]. Leafy vegetables can be contaminated during production[3,4,5], or during post-harvest operations (handling[6], processing[7], etc.) and in 2012 the *Codex Alimentarius Commission* recognized noroviruses as one of the most important microbial risks[8].

Irrigation by wastewater or conventional water can contaminate the surface of plants, even their interior after internalization of viruses *via* the roots and migration without inactivation to the parts eaten raw[9]. Brought to the surface of plants, viruses or virus-like-particles (VLPs) may concentrate in the veins, at the cut ends of plants and around their stomata[10,11]. In the stomata, they could be protected from physical elimination or inactivation

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2 Hall A.J., Eisenbart V.G., Etingüe A.L., Gould L.H., Lopman B.A., Parashar U.D. 2012. Epidemiology of foodborne norovirus outbreaks, United States, 2001–2008. *Emerging infectious diseases*, 18(10), 1566.

3 Stine S.W., Song I., Choi C.Y., Gerba C.P. 2011. Application of Pesticide Sprays to Fresh Produce: A Risk Assessment for Hepatitis A and Salmonella. *Food Environ. Virol.* 3, 86–91.

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often favoured by relative air moistures between about 40 and 70%[12]. Numerous articles have dealt with the transfer of plant pathogenic viruses (phytovirus) within their host (s)[13,14]. After an initial infection, usually of epidermal or mesophyll cells, phytoviruses multiply in infected cells and propagate in the symplasm from cell to cell *via* plasmodesmata, or over longer distances through the phloem reached and then leaved by crossing various cellular barriers (bundle sheath, vascular parenchyma and companion cells involved in the loading of viruses into sieve elements of the phloem). Viruses may be transferred as virions or nucleic acid associated with other proteins; several proteins may be involved (movement proteins, coating proteins, etc.). However, the internalisation of human pathogen enteric viruses and their transfer into a plant is *a priori* in no way comparable: no replication in the plant, internalisation *sensu stricto* preferentially *via* the roots and maybe *via* injuries of aerial parts, passive transfers and almost exclusively in the metaxylem. To our knowledge and excluding reviews, only 13 studies have examined from 1982 to the present day the possibility for human pathogen enteric viruses or their models to be internalized within plants. The very existence of an internalisation of enteric viruses in plants has been controversial: some studies considered it to be limited[15,16], but most have shown rapid internalization (from the first day), with the presence of infectious virus in plant tissues[10,17,18,19,20,21]. The viruses seem to be preferentially transported from the roots to the aerial parts through the xylem[10]. Some studies have shown that enteric viruses can also be internalized from even minor injuries (e.g. caused by manipulation). Virus internalization seems to depend on the plant[20,22], the differences between plants being able to result from differences of transpiration[19], from specific virus/plant interactions[23], or from plant defensive system. It also depends on the virus[19,20],

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probably related to their isoelectric points[24], their surface hydrophobicity[25] and specific receptors[26] that affect their immobilization. Beyond this, it depends on the type of vegetable cultivation[10,21,22,27] (with or without soil, the presence of soil allowing partial immobilization of the viruses[22,28]), the concentration of virus in the contaminating water and the duration of exposure[17,21], and physicochemical conditions[21] (pH, light, soil moisture, relative humidity, temperatures ...).

Our objectives were (i) to characterize the impact of irrigation type on the surface and internalized contaminations of aboveground and underground parts of green onion, and (ii) to describe virus fate of viruses after the initial contamination of the plant.

IV.2. Materials and methods

IV.2.a. Virus, soil, plants and artificial soil solutions

The cytopathic CW1 strain of murine norovirus (MNV-1), as surrogate of human enteric noroviruses[29], was kindly provided by Prof. H.W. Virgin (University of Washington, MO, US). Noroviruses were propagated on RAW 264.7 mouse leukemic monocyte macrophages (ATCC® TIB-71™) as described before[30]. Two days after infection, the cells and their culture medium were collected, frozen in liquid nitrogen and thawed three times, and then centrifuged (10 minutes at 1048 g then 30 minutes at 5869 g). The supernatant was transferred to Amicon® Ultra-15 tangential filters (Merck Millipore LTD, Irlande) and centrifuged for 40 minutes at 1048 g. The retentate was resuspended in 1 mL phosphate-buffered saline (PBS); 14 mL of concentrated suspensions thus obtained were mixed, and the final suspension was aliquoted in 1 mL samples that contained approximately $10^{10.1}$ GC.mL^{-1}, including 10^{9} pfu.mL^{-1}, corresponding to about 8% infectious virus. The 14 aliquots were frozen by immersion in liquid nitrogen and were then stored at -86°C until use. About 10 L of viral suspension was prepared on May 10th, 2016 for contamination of the green onion crop. 10

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24 Michen B., Graule T. 2010. Isoelectric points of viruses. *Journal of Applied Microbiology* 109, 388-397.

25 Dika C., Ly-Chatain M.H., Francius G., Duval J.F.L., Gantzer C. 2013. Non-DLVO adhesion of F-specific RNA bacteriophages to abiotic surfaces: importance of surface roughness, hydrophobic and electrostatic interactions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 435, 178-187.

26 Cromeans T., Park G.W., Costantini V., Lee D., Wang Q., Farkas T., Lee A., Vinjé J. 2014. Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Applied and Environmental Microbiology* 80(18), 5743-5751.

27 Hirneisen K.A., Sharma M., Kniel K.E. 2012. Human enteric pathogen internalization by root uptake into food crops. *Foodborne Pathog. Dis.* 9, 396-405.

28 El Zanati O. 2011. Rétention et restitution d'un Mengovirus murin, utilisé comme substitut du virus de l'hépatite A, dans un modèle de colonne de sol en fonction de la granulométrie des agrégats et propriétés physicochimiques de la solution circulante. Mémoire de Master 2 de l'Université d'Avignon et des Pays de Vaucluse. (stage effectué à l'INRA d'Avignon).

29 Wobus C.E., Karst S.M., Thackray L.B., Chang K.O., Sosnovtsev S.V., Belliot G., Krug A., Mackenzie J.M., Green K.Y., Virgin H.W. 2004. Replication of Norovirus in Cell Culture Reveals a Tropism for Dendritic Cells and Macrophages (Michael Emerman, Ed.). *PLoS Biology*, 2, e432.

30 Belliot G., Lavaux A., Souihel D., Agnello D., Pothier P. 2008. Use of Murine Norovirus as a Surrogate To Evaluate Resistance of Human Norovirus to Disinfectants. *Applied and Environmental Microbiology*, 74, 3315-3318.

aliquots at $10^{10.1}$ GC.mL^{-1} of the viral suspension stored at -86°C were thawed at room temperature for 1 h and then diluted in 10 L of water to obtain contaminated irrigation water "mimicking" contaminated domestic wastewater. Viruses were quantified by RT-qPCR and plaque assay. For RT-qPCR, 60 µL of viral RNA were extracted from 140 µL of solution following the manufacturer instructions (QIAamp® Viral RNA kit (Qiagen)) and immediately purified with One-Step™ PCR Inhibitor Removal (Zymo Research Corp, CA) according manufacturer's protocol. RT-qPCR was performed as previously described[31] using a Mx3005P® qPCR automaton (Agilent Technologies, France). Plaque assays were performed in 10-fold dilutions of virus inoculum as described before[32].

About 1 000 kg of the 0-15 cm surface layer of calcaric phaeozem (FAO classification) were sampled in the Limagne Noire small region near Clermont-Ferrand, France (GPS 45.859 N, 3.201 E). Average annual rainfall was 566 mm for the period 2010-2016 at the nearest climatic station that is 9 km southwest. The field where sampling took place has been irrigated every year by treated wastewater from the wastewater treatment plant of Clermont-Auvergne-Metropole after an additional lagooning tertiary treatment. It has been cultivated with sugar beet since 2015; the soil was bar, recently tilled but without having been sown during sampling on 5 January 2016. The soil was then stored until use at room temperature at a residual moisture of 6.9% dry weight basis. The properties of the 0-15 cm layer were: 3 g.kg^{-1} $CaCO_3$, and after decarbonation, 559 g.kg^{-1} clay, 222 g.kg^{-1} silt, 216 g.kg^{-1} sand, 16.5 g.kg^{-1} organic C, 1.58 g.kg^{-1} total N, 12.7 mg.kg^{-1} N-NH_4^+, 29.9 mg.kg^{-1} N-NO_3^-. Soil pH(water) was 8.16. The concentrations in exchangeable Ca^{2+}, Mg^{2+}, Na^+, K^+, Fe, Mn and Al extracted by cobaltihexamine (NF ISO 23470) were 36.4, 6.25, 0.625, 1.1, 0.0386, 0.013 and 0.14 cmol$^+$.kg^{-1} soil, respectively.

About 200 very young green onion seedlings (*Allium fistulosum*) were produced by *Arom'Antique* plant nursery in Parnans (France) (sown on February 12th, 2016, start of the emergence at the beginning of March, transfer to INRA Avignon on March 15th, 2016 at very young stages (from plants not still emerged to plants with 1-2 stems-leaves.) The young plants were still grown in cavity seedling trays in a greenhouse until they could be transplanted on April 1st, 2016 in planters filled with soil.

Irrigation water was groundwater without enrichment in fertilizers of pesticides.

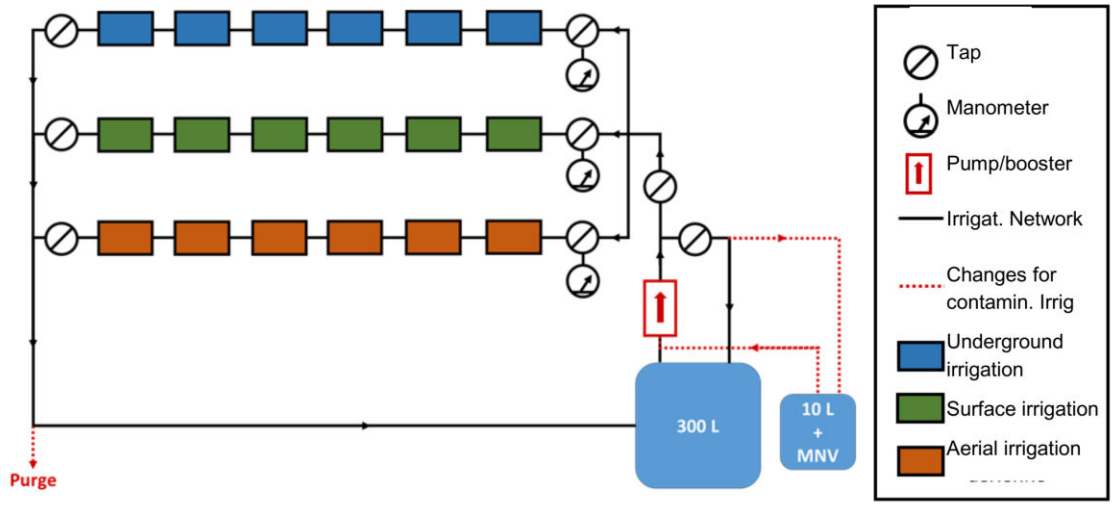
IV.2.b.Experimental design and setup; date and type of sampling

[31] Belliot G., Lavaux A., Souihel D., Agnello D., Pothier P. 2008. Use of Murine Norovirus as a Surrogate To Evaluate Resistance of Human Norovirus to Disinfectants. *Applied and Environmental Microbiology*, 74, 3315-3318.

[32] Wobus C.E. ; Karst S.M., Thackray L.B., Chang K.O., Sosnovtesev S.V., Belliot G., Krug A., Mackenzie J.M., Green K.Y., Virgin H.W. 2004. Replication of Norovirus in Cell Culture Reveals a tropism for Dendritic Cells and Macrophages (Michael Emermean, Ed). *PLoS Biology*, 2, e432.

The experiment was carried out in a CMF greenhouse built in 1995 that was generally used for the cultivation of plants as food for aphids in an INRA insectarium. The greenhouse was equipped with a gas boiler for thermosiphon heating, ridge aeration, cooling boxes, and thermal screen for shading. Its climate was driven by an ARIA® system; the air temperature was recorded by a SIRIUS system.

Eighteen polypropylene planters 89.5 × 33 × 30 cm filled with a calcaric phaeozem were arranged along 3 lines of 6 planters, each line corresponding to one type of irrigation: (i) aerial irrigation from drippers about 40 cm from the soil surface, (ii) surface irrigation from at about 2 cm from the soil surface, and (iii) buried irrigation at about 4 cm below the soil surface (**Figure IV.1** and **Photo IV.1**). 9 young plants were transplanted in each planter along a central line 8 cm apart between successive plants. They were initially abundantly watered to avoid plant death. From the fifth day before the contaminating irrigation, they were irrigated once a day in the morning during 2.5 minutes using aerial, surface or buried drip irrigation by using NDJ Click-Tif PC drippers with 1.1 L.hour^{-1} at about 0.1 MPa water overpressure (corresponding to 46 mL supplied to each plant or 2mm of irrigation water). Water was supplied from a tank (300 L capacity) through a CME 3-4 IKCCD AVBE-97755485 booster (Grundfos, Denmark) and polypropylene tubes. A common return to the 3 lines allowed bringing back water in the tank.



Figures IV.1: Experimental setup used for the monitoring of virus immobilisation.

Line inlet and outlet pressure gauges as well as inlet and outlet valves enabled to ensure approximately 0.01 MPa water overpressure. All irrigations were performed using virus-free groundwater, except for May 10, 2016 where the water was artificially contaminated. On this day, the pump was first disconnected from the main tank and the circuits were purged at low pressure so as to prevent dripping at the level of drippers. The pump was then connected to a 20 L secondary tank filled with about 10 L of the viral suspension, and the irrigation pipes were saturated at low pressure with this suspension. Following the contaminant irrigation, the

irrigation pipes and the booster were rinsed at low water pressure by virus-free groundwater for about 5 min without any return of water to the main reservoir.

Photo IV.1: Experimental setup used for the monitoring of virus immobilisation.

Just before the contaminating irrigation, a wettable sewing thread was fixed to each aerial dripper and left in place until the end of experiment. It wrapped around the plant to ensure that irrigations (contaminating and non-contaminating later) directly reach the plant.

Virus distribution was monitored at six dates: the day before the contamination, approximately 2 h after contamination, and 1, 3, 7 and 10 days later. At each sampling date and for each type of irrigation, 1 planter with its 9 onions was used and condemned. In each planter and for each replicate, the upper and lower halves of the stem-leaves as well as the small young bulbs of onion plants were sampled separately; each of these plant portions was divided into 2 aliquots to elute viruses retained on the plant external surface and extract viruses internalized in the plant tissues, respectively. On the same planters, soil was sampled in the 0-4 cm and 4-20 cm horizons to monitor at the same dates the quantity of viral RNA and soil moisture. In addition, the day after the contamination, a map of soil moisture and viral RNA quantities was performed on a vertical slice of soil for each type of irrigation.

To better understand our results, we monitored simultaneously (i) the virus concentration in the irrigation water, (ii) the soil moisture and temperature, and (iii) at the air temperature and relative moisture within the greenhouse, as well as the global radiation near the planters.

IV.2.c. Elution/extraction of viruses or viral RNA from plants and soil, concentration and purification

The elution and subsequent concentration of the viruses immobilized on the external surfaces of the green onions were performed according to a protocol almost identical to the protocol ISO/TS15216-1[33]. In practice, 7-12 g of the upper part or the lower part of stem-leaves cut into 3-5 cm long pieces or 4-5 intact bulbs were put in a 400 mL Stomacher® filter bag with 40 mL of TGBE (Tris/Glycine/Beef Extract) buffer (100 mM Tris, 50 mM glycine, 1% beef extract) at pH=9.5 and submitted to continuous sway at room temperature for 20 minutes using a SSL3 three-dimensional rocker (Stuart-equipment, Cole-Parmer, Staffordshire, UK) set at 60 rpm. The eluate was collected in a 50 mL conical tube and centrifuged at 10 000 g for 30 minutes at 4°C to clarify the suspension. The supernatant was then transferred to a new conical tube and its pH was adjusted to 7.0 by addition of 1 N HCl. Then, a volume of a solution of PEG-8000 (500 $g.L^{-1}$) and NaCl (87.8 $g.L^{-1}$) was added to the eluate in a ratio 1:4 (ie about 10 mL) to obtain a final concentration of 100 $g.L^{-1}$ of PEG-8000 and 0.3 $mol.L^{-1}$ of NaCl. The samples were then shaken for 60 s using a Vortex at maximum speed and the mixture was stirred during 1 hour using a Reax 2 rotary stirrer (Heidolph-Instruments GmbH, Schwabach, Germany) at a minimum speed. The viral particles, flocculated with PEG 8000, were then sedimented by centrifugation at 10 000 g for 30 minutes at 4°C. After removal of the supernatant, the virus-containing pellet was re-compacted and centrifuged at 10 000 g for 5 minutes at 4°C. The remaining pellet was resuspended in 500 µL of PBS at pH=7.3[34] and separated into three 140 µL aliquots to be stored at -86°C until use.

The extraction of viruses internalized in plants was adapted from the recent work of Di Caprio et al.[35]. After harvest, the viruses immobilized at the surface were destroyed by immersing and stirring the cut plant tissues successively in 30 mL of a solution of sodium hypochlorite (2000 ppm total chlorine) for 5 minutes at room temperature, then rinsing in 30 mL of ultrapure water for 5 minutes, and finally treated with 30 ml of 0.25 M sodium thiosulfate to neutralize the residual chlorine. Total chlorine levels were checked on certain dates. The samples were then milled after freezing in liquid nitrogen with an A11 analytical mill (IKA®-Werke GmbH & Co, Staufen, Germany). The obtained ground material was

[33] ISO. 2012. Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantification. (ISO/TS 15216-1);

[34] Deboosere N., Pinon A., Caudrelier Y., Delobel A., Merle G., Perelle S.? Belliot G. 2012. Adhesion of human pathogenic enteric viruses and surrogate viruses to inert and vegetal food surfaces. *Food microbiology* 32, 48-56.

[35] DiCaprio E., Culbertson D., Li J. 2015. Evidence of the internalization of animal caliciviruses *via* the root of growing strawberry plants and dissemination to the fruit. *Applied and Environmental Microbiology*, AEM-03867.

suspended in 50 mL tubes in 5 mL of PBS at pH=7.0 and stirred for 2 minutes on a vortex. It was then centrifuged at 10 000 for 30 minutes to remove cell debris; the supernatant was transferred into 15 mL conical tubes and centrifuged one time more at 10 000 g for 30 minutes to remove remaining cell debris. The eluate was then aliquoted into three 140 µL samples which were frozen in liquid nitrogen and stored at -80°C.

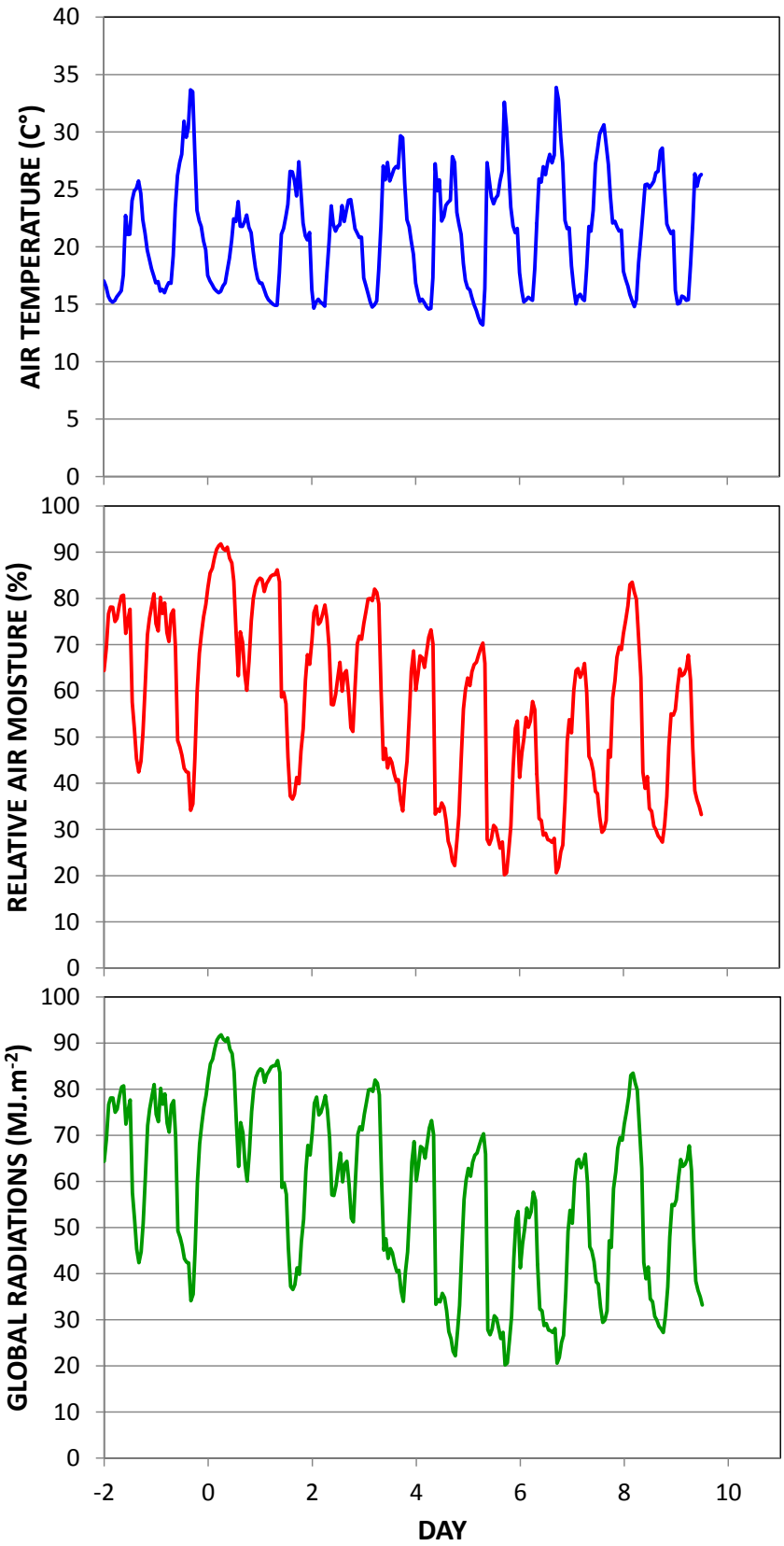
We directly extracted the viral RNA from the soil rather than the whole viruses. The procedure of extraction, concentration and purification was derived from the method proposed by Griffiths et al[36]. As far as possible, we took RNAse-free products and glassware to limit the risk of altering the viral RNA before its quantification. The extractions were carried out on 0.5 g samples of moist soil by addition of 0.5 mL of hexadecyltrimethylammonium bromide (CTAB) as extraction buffer and 0.5 mL of a phenol-chloroform-isoamyl alcohol mixture. (25:24:1) (pH 8.0). The CTAB extraction buffer was prepared by mixing equal volumes of 10% (w/v) CTAB (Sigma, Poole, UK) and pH 8 phosphate buffered saline (PBS) buffer (with 0.7 M NaCl and 240 mM potassium phosphate buffer, pH 8.0). The samples were then lysed by stirring on 100 MiniG® automated tissue homogenizer and cell lyser (SpexSamplePrep, Metuchen, NJ, USA) for 3 cycles of 30 s. The aqueous phase containing the RNA was separated by centrifugation (16 000 g) for 5 minutes at 4°C; it was then sampled and the phenol was removed by mixing with an equal volume of chloroform-isoamyl alcohol (24:1) followed by a new centrifugation (16 000 g) for 5 minutes at 4°C. All the RNA were then floculated by adding to the aqueous solution 2 volumes at 30% (w/v) of polyethylene glycol 6000 (Fluka BioChemika) and 1.6 M NaCl. The mixture was incubated with constant stirring using a stirrer Reax 2 rotary at minimum speed for 2 hours at laboratory temperature; it was then centrifuged (18 000 g) at 4°C for 10 minutes. The pellet of flocculated nucleic acids were then washed in 70% (v/v) ethanol cooled on ice, and air-dried before resuspending in 50 µL of RNase-free Tris-EDTA buffer (pH 7,4) (Severn Biotech, Kidderminster, United Kingdom).

IV.3. Results and discussions

IV.3.a. Greenhouse climate, water supplied during irrigation, water contamination

Temperatures fluctuated between 13 and 34°C from the day before the contaminating irrigation to the last sampling day. Likewise, the air relative moisture fluctuated between 20 and more than 90% (**Figures IV.2**). It has been clearly demonstrated that relative air moistures between about 20 and 75% can cause the quick inactivation of some viruses[12], and that high temperatures can accelerate the phenomenon. Radiation fluctuated but probably did not directly participate to the inactivation of viruses, as virus RNA can be altered by UV radiation, especially C-type UV, which are stopped by greenhouse canopies.

[36] Griffiths R.I., Whiteley A.S., O'Donnell A.G., Bailey M.J. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA-and rRNA-based microbial community composition. *Applied and Environmental Microbiology* 66, 5488-5491.



Figures IV.2a-c: Main characteristics of the greenhouse climate during the sampling period: (a) air temperature, (b) relative air moisture, and (c) global radiation within the greenhouse.

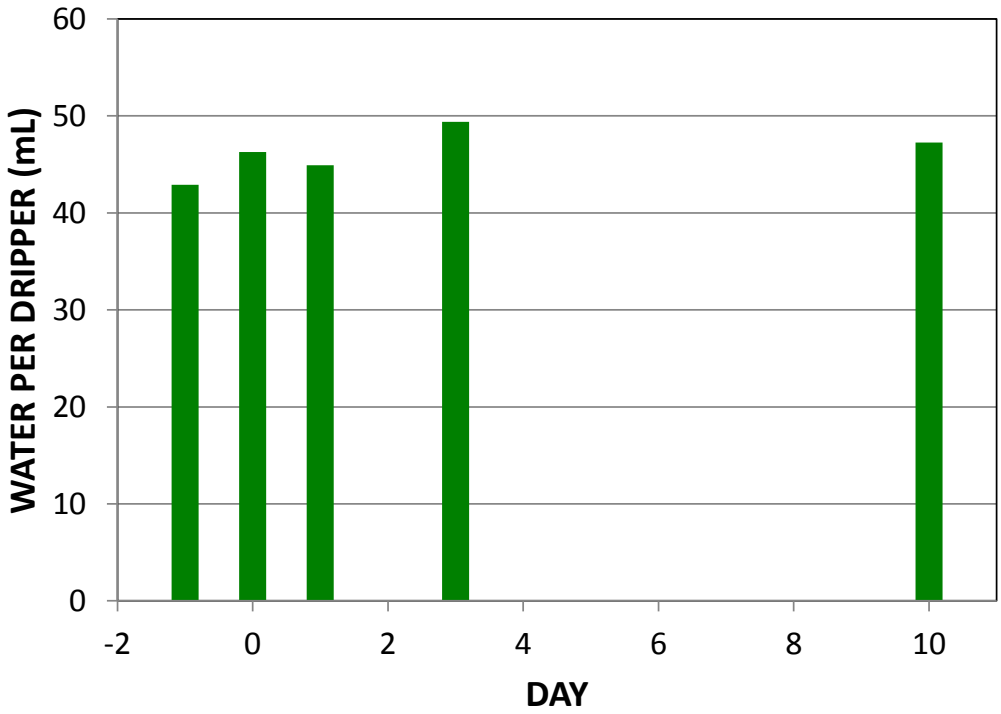


Figure IV.3: Variability of daily water irrigation averaged every day on 9 drippers within the same planter.

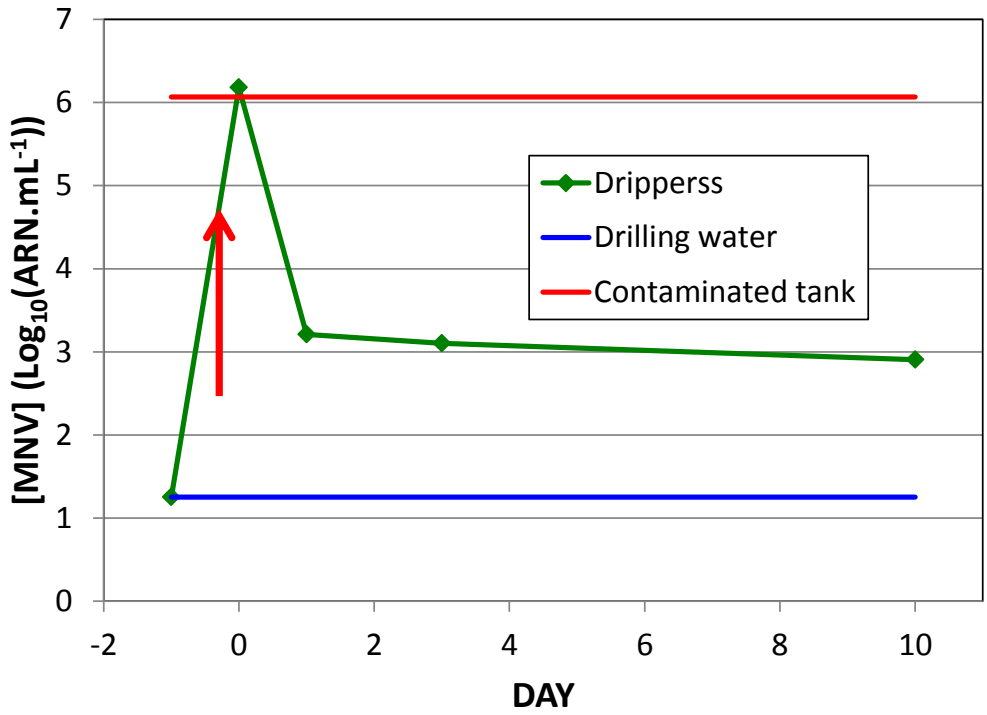


Figure IV.4: Variability of daily water irrigation averaged every day on 9 drippers within the same planter.

An experimental evaluation of the quantities of water supplied was carried out on the day of the irrigation and the following days for the 9 drippers used for the aerial irrigation of the green onions sampled the day before the contaminating irrigation. The results presented in **Figure IV.3** clearly indicate that the irrigation doses were similar from one day to another and close to the calculated value (45.8 mL) for the nominal flow of the drippers.

Since murine norovirus is naturally present in the environment, we characterized its concentration in the irrigation water the day before the contamination, the day of the contaminating irrigation (at the level of the contaminated water supply tank and the level of 9 drippers) and the following days (**Figure IV.4**). Murine noroviruses were naturally present in the drilling water used for irrigation (before its artificial contamination), but well below the concentrations used for the calibration range of RT-qPCR (**Figure IV.4**). Virus concentration in the contaminated tank was identical to that of water coming out of the drippers the day of the contaminating irrigation; similarly, the concentration of murine noroviruses in the dripping water was only one-thousandth of the contaminating concentration the day after the contaminant irrigation.

Our experimental protocol thus enabled to ensure the homogeneity of virus concentrations along the irrigation lines before the beginning of the contaminating irrigation, all the more the concentrations were tested near the end of the line (5th planter in the line devoted to aerial irrigation).And the same procedure enabled to ensure almost perfect rinsing of the irrigation pipes just after the contaminant irrigation. The abatement of 3 $\log_{10}$ suggests a dilution per 1000 of a residue of contaminated water which then remained in the more or less closed circuit. However, we notice some incidents: (1) a significant water leak near one of the buried drippers which certainly increased the soil moisture of this planter and the quantity of viruses in the soil of this planter, and (2) a leak at the level of an aerial dripper that appeared a few days before the end of the experiment.
Thus, about 5.8 10^7 viruses were supplied per onion (for 45.8 mL of water having about $10^{6.1}$ $GC.mL^{-1}$) were provided.

IV.3.b. External and internal onion contaminations

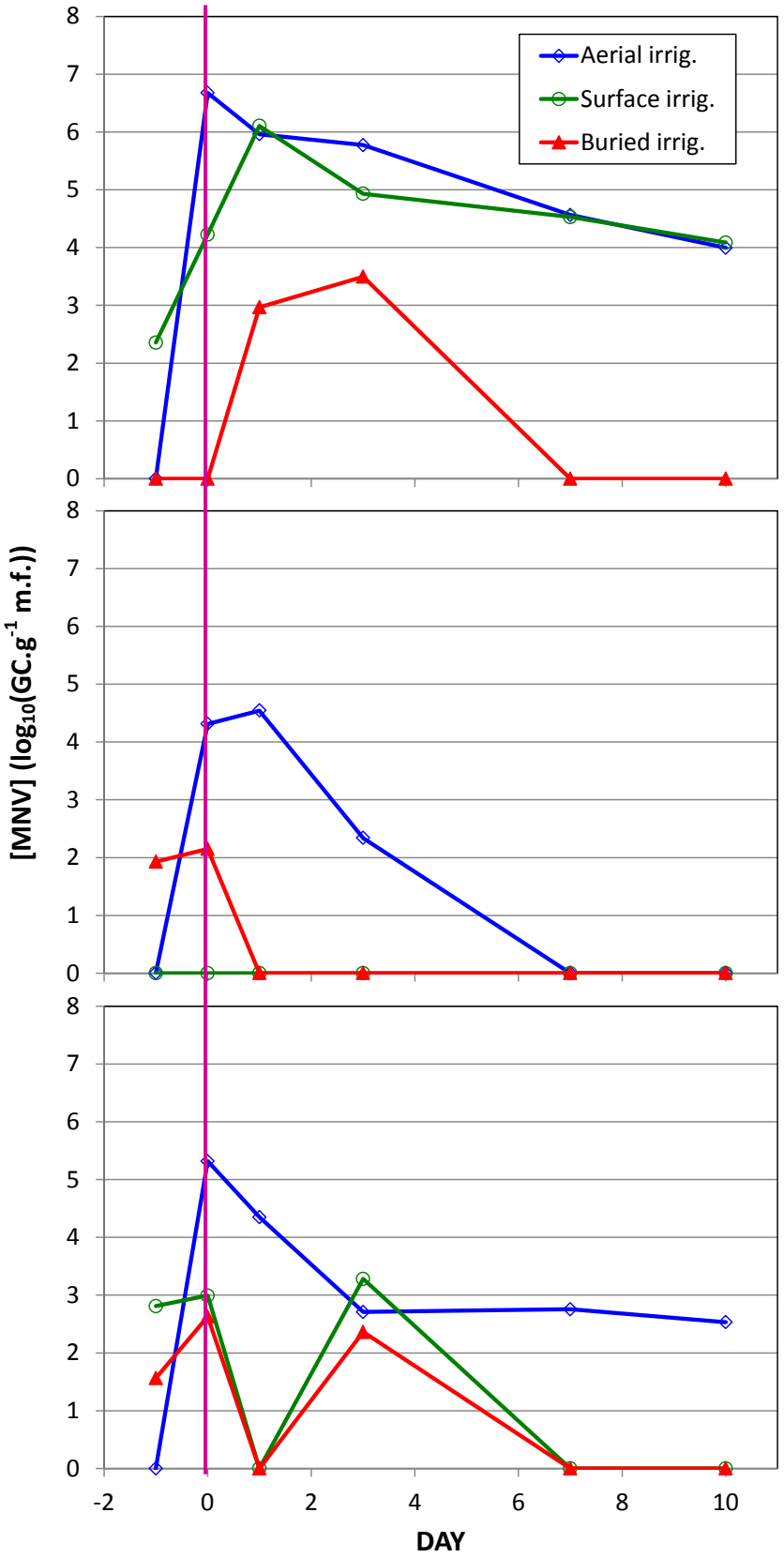
Viral contaminations of the external surfaces of green onions have been reported on **Figures IV.5**.
Aerial and surface irrigations have resulted in very similar or even identical contaminations of the bulbs (**Figure IV.5a**). The level of contamination reached about 10^6-10^7 $GC.g^{-1}$ of fresh onion. As the bulbs were still young and barely trained, these concentrations corresponded to about 4.7 10^6 $GC.bulb^{-1}$, i.e. 8-9% of the quantity of viruses supplied with irrigation. By contrast, underground irrigation induced only a few contamination of the bulbs (about 10^3-10^4 $GC.g^{-1}$ of fresh onion) as the drippers were under the bulbs. Moreover, the absence of surface contamination of the bulbs sampled 7 and 10 days after the contamination probably reflects more the random nature of the contamination when it results from underground

irrigation. Beyond the initial contamination of the bulbs for the aerial and surface irrigations, one then observes a decrease of contamination almost identical for these 2 modes of irrigation that follows an exponential decay, suggesting a disappearance of virus proportional to the amount of remaining viruses and caused by virus-free irrigations after the contaminating day. In our experimental conditions, virus removal was about 0.3 $\log_{10}$ per day.

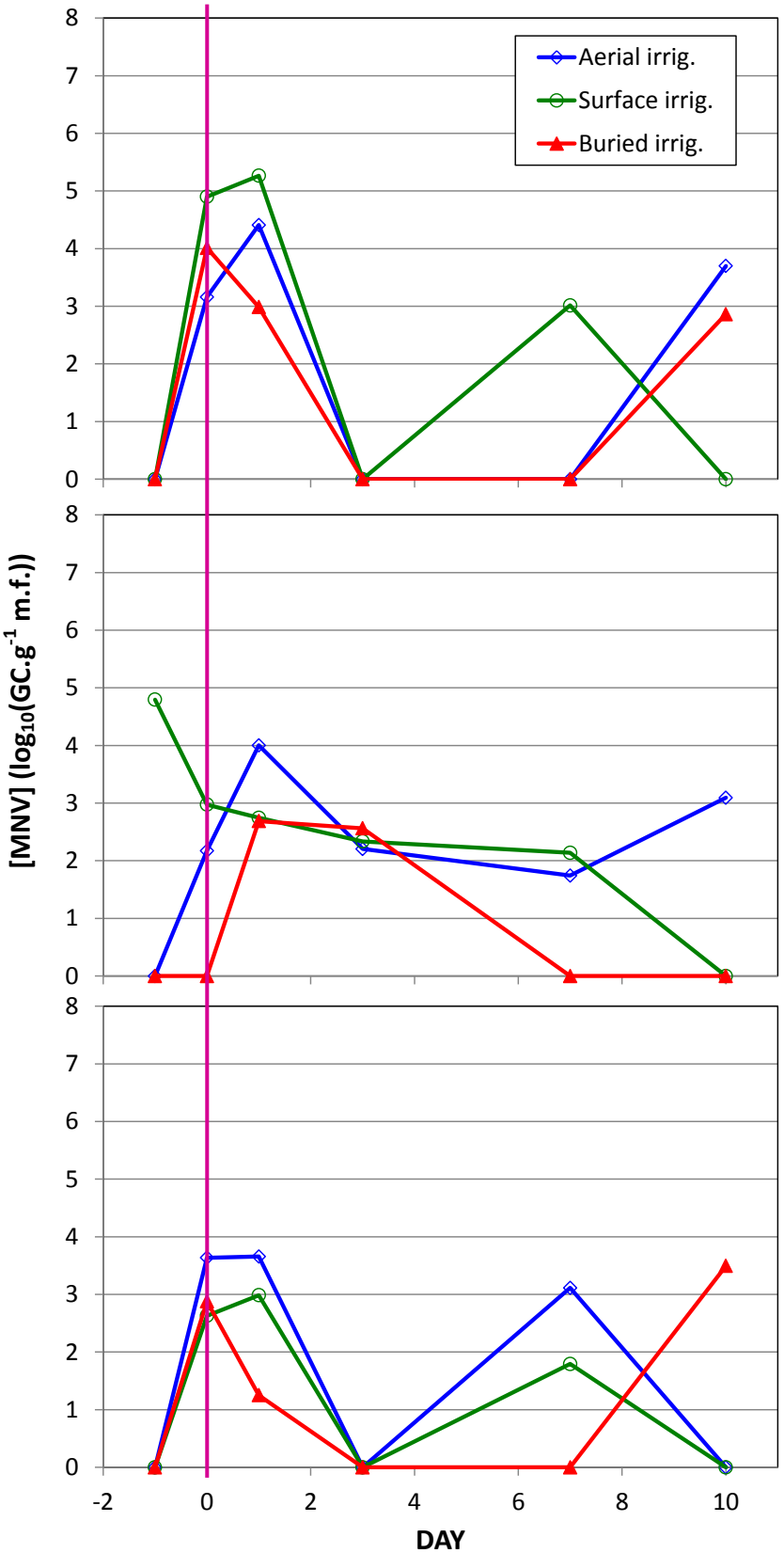
Viral contaminations of the external surfaces of the lower halves of the stem-leaves could *a priori* be neglected for surface and buried irrigations (**Figure IV.5b**), the non-zero values resulting surely from inadvertent contamination either during onion sampling or more probably later during laboratory steps of this work. Moreover, their contaminations (less than or equal to 10^2 GC.g^{-1}) are extremely low (values well below the minimum concentration used to calibrate the RT-qPCR). By contrast, aerial irrigation strongly contaminated the lower halves of the stem-leaves (about 10^4-10^5 GC.g^{-1}), although contamination levels remained lower than those obtained on the bulbs. The peak concentrations achieved amounted to approximately 7.0 10^4 viruses per plant, corresponding approximately to 0.1% of the quantity of viruses initially brought to each plant. The weakness of the levels reached can be explained by the fast transit of the drops of water along the stem-leaves (contrary to the case of bulbs that could be surrounded by stagnant water). In the days following the contaminating irrigation, the decrease of contamination could be described by an exponential decay, with a removal of about 1 $\log_{10}$ per day that was higher than that observed for the bulbs. It could probably be related to the rinsing of the stems by the irrigated irrigation water. However, another track should be mentioned: the greenhouse was equipped with a powerful fan that participate to the temperature regulation; this fan may have caused the partial aerosolization of viruses (even if this fan did not blow directly on our "table" and the aerial irrigation line was at the "table" edge opposite to the fan and close to the glass walls of the greenhouse that can slow down the wind).

Viral contamination of the external surfaces of the upper halves of the stem-leaves was observed for aerial irrigation with a maximum of about 10^5-10^6 GC.g^{-1} (**Figure IV.5c**); this level was similar or slightly higher than that for the lower stem-leaves. The decrease rate of the contamination was initially similar to that for the halves lower stem-leaves during the first 3 days following contamination. Beyond this date, contamination seemed to stabilize. Punctual contaminations for surface and buried irrigations surely resulted from involuntary contaminations in the greenhouse or the laboratory. It should be noted that onion stem-leaves sometimes bent and could touch the soil surface; the phenomenon was probably more pronounced for the so-called surface irrigation, for which the stem-leaves sometimes had to make their way around the dripper pipe placed a few cm above the surface of the soil.

Viral contaminations within green onion tissues after internalizations have been reported on **Figures IV.6a-c**.



Figures IV.5a-c: Evolution of the contamination of the external surfaces of (a) the bulbs, (b) the lower halves of the stem-leaves, and (c) the upper halves of the stem-leaves. *(The purple vertical bar corresponds to the date of contamination).*



Figures IV.6a-c: Evolution of the internalized of (a) the bulbs, (b) the lower halves of the stem-leaves, and (c) the upper halves of the stem-leaves. *(The purple vertical bar corresponds to the date of contamination).*

Concerning the bulbs (**Figure IV.6a**), virus internalization seemed to be similar for the three types of irrigation during the first 3 days after the contaminant irrigation. In particular, there is *a priori* no difference between internalization for buried irrigation and internalization for drippers above the soil surface. The lack of difference probably results from a good colonization of the soil by the roots (results not shown) allowing the green onion plants to absorb water (and viruses) coming from the drippers whatever their position. The maximum levels of contamination were approximately 10 times lower than the levels of external surface contaminations (when expressed per g of fresh product). On the 3rd day after the contaminating irrigation, we could no longer detect viruses internalized within bulbs; their disappearance suggests their migration by convection with the flow of raw sap. The concentrations observed 7 and 10 days after the contaminating irrigation could have resulted from sample contamination at any stage of the experiment between plant sampling and final RT-qPCR analysis.

Concerning the lower halves of the stem-leaves (**Figure IV.6b**) and ignoring an outlier value for surface irrigation the day before the contamination, we observed as a first approximation a similar internalizations of viruses for the three types of irrigations with a maximum reached on the day of the contamination (surface irrigation and aerial irrigation) or the following day (buried irrigation) reaching 10^3-10^4 GC.g^{-1}. This contamination seems more durable over time than the contamination of the bulbs with a slow decrease in concentrations with time (virus removal between 0.2 and 0.9 $\log_{10}$ per day).

Concerning the upper halves of the stem-leaves (**Figure IV.6c**), we observed similar internalizations for the three types of irrigations up to 3 days after the contamination. The maximum concentrations reached the day of the contamination and/or the next day were approximately 10^3-10^4 GC.g^{-1}, *i.e.* values similar to those obtained for the lower halves of the stem-leaves. Beyond the third day after infection, the measurements seemed to diverge with zero values suggesting a rapid disappearance of the viruses, but also values slightly lower than those of the day of the contamination or the next day.

Thus, it remains difficult to conclude whether the internalized viruses disappeared rapidly or not from the lower parts of the plants by convection with the flow of raw sap and, if so, whether they can accumulate at the end of the metaxylem, or if they can to be expelled with transpiration or with guttation. Other questions included the possible effect of essential oils detected in green onions on

IV.4. Conclusions and perspectives

The objectives of this work were (i) to evaluate the impact of green onion irrigation on the initial distribution of viruses in the soil and plant, and (ii) to characterize the subsequent fate of the viruses introduced (transfers and inactivation).

While surface contamination greatly depends on the type of irrigation (i.e. aerial, surface and buried drip irrigation), internalized contamination was as a first approximation independent of the type of irrigation. After the initial contamination, viruses immobilized at

the surface of the bulbs and stem-leaves could be progressively eluted with subsequent virus-free irrigation. Virus removal could be described by an exponential decay, suggesting that it was proportional to the quantity of viruses remaining immobilized and to the irrigation amount. The existence of viruses still internalized beyond the first 3 days after contamination was less certain, and we still have to work on their fate within plants, as wastewater management (standards), agricultural practices (minimum delay between the last contaminating irrigation and the harvest …) and post-harvest treatment of products (washing, removal of certain portions of plants) should be optimized to actual health risks.

Until now, we couldn't assess the proportions of viruses that were still infectious, for various reasons including probably the presence of essential oils that affect RAW cell cultures and maybe viruses themselves[37]. However, it is probable that viruses immobilized on the surface of stem-leaves of green onions were inactivated as a consequence of the relative air moisture values; the inactivation of viruses at the surface of onion bulbs would depend on the relative moisture of the soil gas phase that may be saturated as long as soil aggregates remained saturated. It is unclear whether internalized viruses remained infectious or not, as another work[38] has already noted the internalization of murine noroviruses within the same green onions (*Allium fistulosum*) using plaque assay to quantify virus quantities in the plant tissues.

[37] Seo D.J., Choi C. 2017. Inhibition of Murine Norovirus and Feline Calicivirus by Edible Herbal Extracts. *Food and Environmental Virology*, 9(1), 35-44.

[38] Hirneisen K.A., Kniel K.E. 2013. Inactivation of internalized and surface contaminated enteric viruses in green onions. *International Journal of Food Microbiology*, 166(2), 201-206.

Annexe II

Vomiting symptom of acute-gastroenteritis estimated from epidemiologic data can help to assess river contamination by human pathogen enteric viruses

* Cette Annexe correspond au Chapitre II de la présente thèse tel qu'il a été soumis à *Environmental Science & Technology*. Il contient le manuscrit de l'article accompagné des figures et *Supporting Informations*.

Vomiting symptom of acute gastroenteritis estimated from epidemiological data can help predict river contamination by human pathogenic enteric viruses

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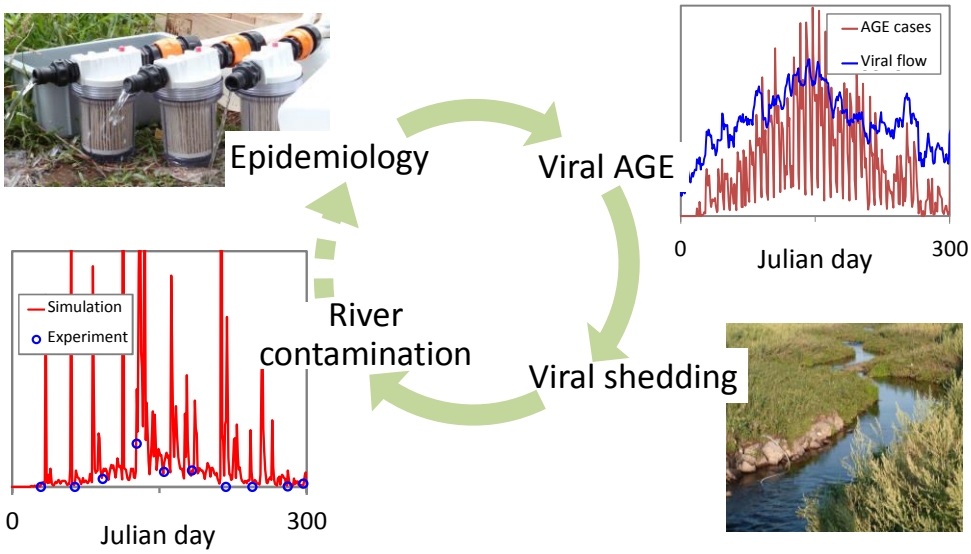
KEYWORDS: Human enteric viruses, acute gastroenteritis, epidemiology, shedding, wastewater treatment plant, river contamination, environmental fate, model.

16 **SYNOPSIS**



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ABSTRACT. Contamination of conventional water bodies by human enteric viruses coming from wastewater discharge is a well-established phenomenon. Here we propose a model of viral contamination of rivers based on acute gastroenteritis epidemiology and assess whether it can simulate in situ experimental monitoring. Noroviruses, rotaviruses, enteroviruses, adenoviruses and hepatitis A viruses were quantified by molecular methods after water concentration. Water flows were obtained from the Hydro databank and the wastewater treatment plant (WWTP) data. Acute gastroenteritis cases based on medical prescriptions were provided by *Santé Publique France*. We estimated the total number of daily viral acute gastroenteritis cases and modeled virus shedding and fate in the WWTP and rivers. Simulated virus concentrations were compared to the weighted sum of measured concentrations. Seasonal variations in viral acute gastroenteritis were predicted from vomiting criteria. All viruses except hepatitis A virus were widely detected in wastewaters and river, in concentrations reaching 10^{+8} $GC.L^{-1}$ for adenoviruses in the Artière River. We were able to predict virus load in raw wastewater and in the Artière River. Estimated weighting coefficients showed the high impact of noroviruses GII. This model can thus serve to compare water treatment, discharge and reuse scenarios.

36 **1. INTRODUCTION**

37 Conventional water resources are increasingly scarce and increasingly polluted, including from
38 contamination by human pathogenic enteric viruses[1–6]. Viral contamination of urban rivers
39 results essentially from wastewater discharges[3] from sewerage systems and wastewater treatment
40 plants (WWTP)[7]. Improved sanitation, which had already reached 40% of the global population
41 in 2010[7], drives wastewater reclamation[8,9]. Wastewater management has to secure sanitary
42 safety. Models combining the epidemiology of viral acute gastroenteritis (AGE), shedding and
43 fate in sewers, WWTP and rivers could help assess health risks of different wastewater
44 management scenarios.

45 To our knowledge, three models have sought to predict enteric virus contamination of rivers[10–
46 12]: Mohammed et al.[11,12] proposed two similar black box models based on environmental
47 variables unrelated to AGE, whereas Petterson et al.[10] combined, for a Norwegian river, its
48 potential norovirus contamination estimated from fecal indicators with local prevalence data on
49 confirmed norovirus AGE cases in Sweden. This second approach was limited by variations in
50 fecal indicator organisms in treated wastewater and their environmental fate, and by possible
51 differences in norovirus prevalence between the diagnosed foreign subpopulation and the whole
52 sick population. As pathogens are rarely identified via stool specimens, estimating the number of
53 viral AGE cases requires symptoms statistically discriminating viral-etiology AGE from others
54 (the most discriminating symptoms being vomiting, fever and mucous diarrhea[13]), then
55 estimating the proportions of these symptoms in the global sick population from a well-known
56 subpopulation (e.g. diagnosed or consulting a physician) and the impact of these symptoms on
57 the probability of being a membership of this subpopulation[14], and then the proportion of viral
58 AGE in the whole population. The proportions of asymptomatic carriers and the kinetics of virus

59 shedding are not well known for each virus species, but quantitative results exist for human
60 noroviruses[15–21] that are the leading cause of AGE[22–25], and Teunis et al.[21] proposed a stochastic
61 model of virus shedding for symptomatic and asymptomatic norovirus GII.4 carriers. The
62 subsequent fate of viruses in sewers, WWTP and rivers spans transport, immobilization and
63 inactivation. In sewers, virus concentrations may be affected by leakages[26,27], dilution with
64 stormwaters, reducing conditions and iron oxides[28], and wastewater residence time that often
65 does not exceed a few hours[29,30]. In rivers, virus removal may be neglected over a few hours, in
66 which time a virus can travel a few km[31]. For combined sewer systems collecting stormwaters,
67 WWTP bypass discharges raw wastewater into the environment. Several meta-analyses[32,33] and
68 reviews[34,35] have dealt with virus removal in WWTPs, and one study has compared a model
69 against experimental data[36]. Classical pre-treatments and activated sludge WWTP treatments
70 generally enable between < 1 $\log_{10}$[37] and 3-4 $\log_{10}$[34] reduction in human enteric viruses, but virus
71 removal is very virus-dependent[38]. Tertiary physical treatments and/or chemical treatments
72 enable additional virus removal[35].
73 Our objectives here were to (i) propose a model of viral contamination of water combining
74 AGE epidemiology, virus shedding and virus fate in the 'sewers–WWTP–surface water'
75 continuum, and (ii) assess whether this model correctly predicts *in situ* contamination in a small
76 region of France.
77

78 ## 2. MATERIALS AND METHODS

79 **Sampling zone and climate context.** The work was carried out on a small geographical
80 area near the city of Clermont-Ferrand, France (45.77 N, 3.09 E) combining the collection basin
81 of wastewaters conveyed from 19 towns and cities (237,700 total inhabitants in 2014) to the

82 *'Trois Rivières'* WWTP, the Artière River in which treated wastewaters are partly discharged but
83 receiving upstream treated wastewater from a small WWTP (120 population equivalents), and a
84 1500-ha area irrigated by the other part of treated wastewaters (representing 30–40% of WWTP
85 effluent flow rate during the irrigation period[39]) after an additional lagooning (Figure S1). The
86 *'Trois Rivières'* WWTP was designed for 425,000 population equivalents. Its treatment train
87 includes a pre-treatment (screening followed by sand and oil removals), a conventional very-
88 low-load biological activated sludge process with enhanced nitrification/denitrification, and a
89 clarification step during which iron chlorosulfate is used to precipitate phosphate.
90 Climate is semi-continental. From September 2015 to August 2016, cumulated rainfall was
91 564 mm, and mean temperature was 12.3°C at the Clermont-Ferrand airport climate station.
92 Additional data concerning the Artière River flow upstream of wastewater discharge (45.77 N,
93 3.14 E) was obtained from the Hydro databank run by the French Ministry for Ecology, Energy
94 and Sustainable Development[40]. Data concerning wastewater flows at the inlet and outlet of the
95 WWTP were provided by the WWTP. We assumed that the Artière River flow downstream of
96 wastewater discharge was equal to the sum of the Artière River flow upstream of discharge, the
97 treated wastewater flow at the WWTP outlet minus water withdrawal for filling the lagoons, and
98 the WWTP by-pass flow. During the same period (September 2015 to August 2016), Artière
99 river flow upstream of the treated wastewater discharge varied between 4320 and
100 140,832 $m^3.day^{-1}$ and was generally more than doubled by treated wastewater discharge (that
101 varied between 29,007 and 144,467 $m^3.day^{-1}$). The mean downstream Artière flow–to–upstream
102 Artière flow ratio was equal to 5.13. During the same annual period, the WWTP by-pass was
103 operated for 60 days with a maximum discharge flow of 10,500 $m^3.day^{-1}$.

104 **Experimental design and protocols.** The experimental monitoring was carried out
105 between 1st June 2015 and 31 August 2016 once a month and once every two weeks outside the
106 period of irrigation by treated wastewater and during this period, respectively. We sampled raw
107 wastewater just after screening (i.e. before sand and oil removal), treated wastewater at the
108 WWTP outlet, and Artière River water upstream and downstream of the WWTP discharge
109 (Figure S1). The temperature and the pH of these waters were measured in the same time.
110 Environmental concentrations of noroviruses (genogroup I, NVGI; genogroup II, NVGII),
111 rotaviruses A (RV-A), enteroviruses (EV), adenoviruses (AdV) and hepatitis A viruses (HAV)
112 were monitored using practically the same water sampling–virus concentration–purification
113 procedure as in Wurtzer et al.[41] based on US Environmental Protection Agency method 1615[42,43].
114 Briefly, water samples were concentrated by three successive steps. River samples (100 L)
115 were filtered *in situ* using electropositive filters (NanoCeram® VS2.5-5, Argonide Corporation,
116 Sanford, FL) at a rate of 2 L. minute^{-1}, after which the filters were returned to the lab, and
117 wastewater samples (1 L) were filtered in-lab using disc filters (NanoCeram® 90MM NC Disk
118 NL, Argonide) at a rate ensuring approximately the same flux of water as for *in situ* filtration.
119 Viruses were then eluted by sonicating for 1 hour the filters immersed in 450 mL (river water
120 samples) or 50 mL (wastewater samples) of a beef extract elution buffer adjusted to pH=9.5 and
121 kept below 10°C. The buffer solutions with eluted viruses were then recovered and their pH was
122 adjusted to 3.5. They were then gently stirred during 1 hour at laboratory temperature before
123 being centrifuged. After centrifugation, the pellets were resuspended in 8 mL phosphate buffer
124 pH=9.0, and the resulting viral suspension was successively clarified by centrifugation and
125 filtration on low-binding 0.45 µm filters. The suspensions were concentrated one last time and

126 their beef extract partly removed by ultracentrifugation at 143,000 *g* for 2 h at 4°C on a 40%
127 sucrose cushion. The final pellet was resuspended in the remaining 0.6 mL sucrose cushion.
128 Molecular detection and quantification of virus genome copies (GC) was performed according
129 to Prevost et al.[3] but with an additional PCR inhibitor removal step using a One-Step PCR
130 inhibitor removal kit (ref. D6030, Zymo Research, Irvine, CA) following the manufacturer's
131 instructions. Primers and probes employed for molecular detection were designed as previously
132 described[3], except for the NVGI probe which was corrected as follows: 5'-FAM-
133 CGA{T}CYCC{T}GTC{C}ACA-BHQ1-3' (the base between brackets corresponds to LNA
134 base). Detection thresholds were about $10^{2.1}$, $10^{0.4}$, $10^{2.2}$, $10^{0.5}$, $10^{2.6}$ and $10^{1.6}$ $GC.L^{-1}$ for NVGI,
135 NVGII, RV-A, EV, AdV and HAV, respectively. When 1 L of water was sampled, these
136 thresholds were a hundred times higher.
137 Additional water aliquots were sampled to quantify *Escherichia coli* (500 mL) at the Vaucluse
138 departmental analysis lab in Avignon (France), total organic C and N (180 mL), anions and
139 cations (200 mL), and NH_4^+ (40 mL).
140 **Epidemiological data and probabilistic estimation of viral AGE incidence.** AGE
141 cases based on medical prescriptions were provided by *Santé Publique France* for the 19 cities
142 connected to the WWTP. For each declared AGE case, information includes age bracket (1–4, 5–
143 65 or >65 yrs), place of residence, and medical prescription (antiemetic, antidiarrheal, antiemetic
144 and antidiarrheal, oral rehydration solution (ORS) for children aged under 15 yrs, and 'Others',
145 i.e. antispasmodics and intestinal adsorbents). Note that antiemetics are contraindicated and
146 rarely prescribed for children in France[44,45]. In 2014, the age-bracket stratification of inhabitants
147 was 12,100 aged 1–4 yrs, 184,600 aged 5–65 yrs and 41,000 aged >65 yrs.

148 We estimated daily number of new viral AGE cases $n_{\mathrm{AGE}}(\mathrm{v},d)$ during the Julian day d for all of

149 these inhabitants from the total number of new viral AGE cases $n_{\mathrm{AGE}}(d)$ and the proportion of

150 new viral AGE cases $p_{\mathrm{AGE}}(\mathrm{v},d)$:

151 $$n_{\mathrm{AGE}}(\mathrm{v},d) = p_{\mathrm{AGE}}(\mathrm{v},d) \times n_{\mathrm{AGE}}(d) \qquad (1)$$

152 $n_{\mathrm{AGE}}(d)$ was estimated from the total number of AGE cases based on medical prescriptions

153 $n_{\mathrm{AGE}}(\mathrm{prscr.},d)$ and the probability of people with AGE presenting to a physician $p_{\mathrm{AGE}}(\mathrm{prscr.})$:

154 $$n_{\mathrm{AGE}}(d) = n_{\mathrm{AGE}}(\mathrm{prscr.},d) \times \left(\frac{1}{p_{\mathrm{AGE}}(\mathrm{prscr.})}\right) \qquad (2)$$

155 $p_{\mathrm{AGE}}(\mathrm{prscr.})$ was set at 0.334[14]. We assumed that daily proportion of viral-etiology $p_{\mathrm{AGE}}(\mathrm{v},d)$

156 among the new daily AGE cases could be estimated from the daily proportion of sick people

157 with vomiting symptoms $p_{\mathrm{AGE}}(\mathrm{vom.},d)$. As long as $p_{\mathrm{AGE}}(\mathrm{vom.},d)$ is between the mean

158 probabilities $p_{\mathrm{AGE|v}}(\mathrm{vom.})$ and $p_{\mathrm{AGE|b}}(\mathrm{vom.})$ of vomiting symptoms for sick people infected by

159 viruses and bacteria, respectively, and implicitly neglecting other possible etiologies, then

160 $p_{\mathrm{AGE}}(\mathrm{v},d)$ can be estimated by the following equation:

161 $$p_{\mathrm{AGE}}(\mathrm{v},d) = \frac{p_{\mathrm{AGE}}(\mathrm{vom.},d) - p_{\mathrm{AGE|b}}(\mathrm{vom.})}{p_{\mathrm{AGE|v}}(\mathrm{vom.}) - p_{\mathrm{AGE|b}}(\mathrm{vom.})} \qquad (3)$$

162 $p_{\mathrm{AGE|b}}(\mathrm{vom.})$ and $p_{\mathrm{AGE|v}}(\mathrm{vom.})$ were set at 0.52 and 0.66, respectively[13]. $p_{\mathrm{AGE}}(\mathrm{v},d)$ was set at

163 0 for $p_{\mathrm{AGE}}(\mathrm{vom.},d) \leq p_{\mathrm{AGE|b}}(\mathrm{vom.})$ and at 1 for $p_{\mathrm{AGE|v}}(\mathrm{vom.}) \leq p_{\mathrm{AGE}}(\mathrm{vom.},d)$.

164 $p_{\mathrm{AGE}}(\mathrm{vom.},d)$ was estimated from the corresponding daily proportion of vomiting symptoms

165 among new cases of AGE-related prescriptions $p_{\mathrm{AGE|prscr.}}(\mathrm{vom.},d)$:

166 $$p_{\mathrm{AGE}}(\mathrm{vom.},d) = \frac{-p_{\mathrm{AGE|prscr.}}(\mathrm{vom.},d) \times p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.})}{p_{\mathrm{AGE|prscr.}}(\mathrm{vom.},d) \times [p_{\mathrm{AGE|vom.}}(\mathrm{prscr.}) - p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.})] - p_{\mathrm{AGE|vom.}}(\mathrm{prscr.})} \qquad (4)$$

167 where $p_{\mathrm{AGE|vom.}}(\mathrm{prscr.})$ and $p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.})$ are the mean probabilities to present to a

168 physician when suffering from vomiting symptoms or not, respectively. Two alternative

169 hypotheses were used to estimate $p_{\mathrm{AGE|vom.}}(\mathrm{prscr.})$ and $p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.})$:

- $p_{\mathrm{AGE|vom.}}(\mathrm{prscr.})$ and $p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.})$ were equal to $p_{\mathrm{AGE}}(\mathrm{prscr.})$, as Van Cauteren et al.[14] did not include vomiting and nausea symptoms as determinants of physician consultation in their final multivariate logistic regression;
- $p_{\mathrm{AGE|vom.}}(\mathrm{prscr.})$ and $p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.})$ were estimated from the equations for conditional probability:

$$p_{\mathrm{AGE|vom.}}(\mathrm{prscr.}) = \frac{p_{\mathrm{AGE}}(\mathrm{prscr.})}{p_{\mathrm{AGE}}(\mathrm{vom.})} \times p_{\mathrm{AGE|prscr.}}(\mathrm{vom.}) \qquad (5a)$$

$$p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.}) = \frac{p_{\mathrm{AGE}}(\mathrm{prscr.})}{p_{\mathrm{AGE}}(\overline{\mathrm{vom.}})} \times p_{\mathrm{AGE|prscr.}}(\overline{\mathrm{vom.}}) \qquad (5b)$$

where $p_{\mathrm{AGE}}(\mathrm{vom.})$ is mean probability of people with AGE experiencing vomiting. $p_{\mathrm{AGE}}(\mathrm{vom.})$ was set at 0.629[14]. $p_{\mathrm{AGE|prscr.}}(\mathrm{vom.})$ and $p_{\mathrm{AGE|prscr.}}(\overline{\mathrm{vom.}})$ were estimated from epidemiological data between 1st September 2015 and 31st August 2016 (0.79 and 0.21, respectively). $p_{\mathrm{AGE|vom.}}(\mathrm{prscr.})$ and $p_{\mathrm{AGE|\overline{vom.}}}(\mathrm{prscr.})$ were estimated as 0.419 and 0.190, respectively.

Viral shedding, transport and removal modeling. The daily quantity of viruses shed from one symptomatic carrier $Q_{\text{p-sympt.}}(\mathrm{v},t)$ (GC.day^{-1}.people^{-1}) was estimated from virus concentration $C(t)$ (GC.g^{-1} stool) within stools and daily quantity of stool $m_{\text{s-sympt.}}(t)$ (g.day^{-1}) excreted:

$$Q_{\mathrm{p-sympt}}(\mathrm{v},t) = C(t) \times m_{\mathrm{s-sympt.}}(t) \qquad (6)$$

where t is time (day) after onset of infection. $m_{\text{s-sympt.}}(t)$ was set at 500 g.day^{-1} during the first 3 days of AGE, then at 100 g.day^{-1} thereafter[10]. Assuming that virus concentrations in stools did not distinguish symptomatic from asymptomatic carriers[21] and ignoring the variability between infected people, the daily quantity of viruses shed for a symptomatic carrier and the corresponding proportion of asymptomatic carriers $Q_{\mathrm{p}}(\mathrm{v},t)$ (GC.day^{-1}.p^{-1}) is then:

192 $$Q_{\mathrm{p}}(\mathrm{v},t) = C(t) \times \left(m_{\mathrm{s-sympt.}}(t) + \left(\mathrm{m}_{\mathrm{s-asympt.}}(t) \times \left(\frac{p(\mathrm{asympt.})}{1-p(\mathrm{asympt.})} \right) \right) \right) \quad (7)$$

193 where $m_{\text{s-asympt}}(t)$ is daily quantity of stool for asymptomatic carriers (g.day^{-1}), and p(asympt.) is
194 proportion of asymptomatic carriers among infected people. $m_{\text{s-asympt.}}(t)$ was set at 100 g.day^{-1} all
195 the time[10]. Although proportion of asymptomatic carriers and virus shedding are virus-
196 dependent[46–48], we used the data for NVGII that explained most of the experimental trends[49]:
197 p(asympt.) was set at 0.30[21], and virus shedding of symptomatic and asymptomatic carriers was
198 described by the model of Teunis et al.[21]:

199 $$C(t) = C_{\max} \times \mathrm{fac} \times e^{-\alpha \times t} \times \left(1 - e^{-(\beta-\alpha)\times t}\right) \quad (8)$$

200 where $C_{\max}$ is maximum virus concentration reached (GC.g^{-1} stool), and α (day^{-1}), β (day^{-1}) and
201 fac are three interlinked constants as proposed by Teunis et al.[21]. $C_{\max}$, α, β and fac values were
202 set at 10^{+7} GC.g^{-1} stool, 0.53 day^{-1}, 0.48 day^{-1} and –27.2, respectively, in order to fit with the
203 median relationship proposed by Teunis et al.[21]. We neglected virus removal in the sewerage
204 system and the Artière River. Daily flow of viruses at the WWTP inlet, i.e. $Q(\mathrm{v},t)$ (GC.day^{-1}),
205 could then be estimated with the following equation:

206 $$Q(\mathrm{v},d) = \sum_{t=0}^{n_{\mathrm{day}}} Q_{\mathrm{p}}(\mathrm{v},t) \times n_{\mathrm{AGE}}\left(\mathrm{v}, d + d_{\mathrm{prscr.}} - d_{\mathrm{sew}} - t\right) \quad (9)$$

207 where n_{day} is duration of virus shedding (40 day), $d_{\text{prscr.}}$ is delay from onset of shedding to
208 physician consultation (day), and d_{sew} is sewer residence time (day). As average $d_{\text{prscr.}}$ was about
209 1.5 days[14] and d_{sew} is often no longer than a few hours[29,30], we neglected the difference $d_{\text{prscr.}}$ –
210 d_{sew}.

211 Virus removal in the WWTP was simulated in the pretreatment basins, the three aeration zones
212 of the activated sludge basins, and the clarifiers, assuming in each basin the mixture of incoming
213 water with water already present and a first-order kinetic varying with wastewater temperature

214 according to a Q_{10} law (Supporting Information). The simulation of virus concentrations in the
215 Artière River downstream of the wastewater discharge took into account the mixing of treated
216 wastewater with Artière River water, the discharge of raw wastewater during by-pass periods,
217 and the withdrawal of treated wastewater to fill the lagoons used for crop irrigation. By doing
218 this, we neglected other contributions to Artière River flows, including small stream
219 contributions (mainly Le Bec stream), surface runoff during rainy periods, and exchanges
220 between rivers and alluvial aquifers.

221 **Indirect comparison between simulated virus fate and experimental virus**
222 **concentrations.** To enable comparisons between virus concentrations simulated without
223 distinguishing viral species and experimental data for various enteric viruses, we defined
224 equivalent experimental virus concentrations $[v_{eq},d]$ ($GC.L^{-1}$) from the measured concentrations
225 at date *d*:

226 $$[\mathrm{V_{eq}}, d] = \mathrm{a} \times [\mathrm{NVGI}, d] + \mathrm{b} \times [\mathrm{NoVGII}, d] + \mathrm{c} \times [\mathrm{RV\text{-}A}, d] + \mathrm{d} \times [\mathrm{EV}, d] + \mathrm{e} \times [\mathrm{AdV}, d]$$
227 (10)

228 where [NVGI,*d*], [NVGII,*d*], [RV-A,*d*], [EV,*d*] and [AdV,*d*] are the concentrations ($GC.L^{-1}$) of
229 NVGI, NVGII, RV, EV and AdV, respectively, and a, b, c, d and e are five weighting
230 coefficients that express their relative impact on AGE. These coefficients were estimated by
231 fitting equivalent virus concentrations to simulated virus concentrations in raw wastewater, by
232 minimizing the sum of square differences between viral concentrations simulated from
233 epidemiological data and equivalent virus concentrations derived from equation (10). The same
234 coefficients were used thereafter to compare measured virus concentrations against simulated
235 virus concentrations in the Artière River.

236

237 **3. RESULTS AND DISCUSSION**

238 **Number of viral AGE cases estimated from epidemiological data.** Approximately
239 one tenth of the 237,700 inhabitants of the wastewater collection basin consulted a physician due
240 to AGE over the period 1st September 2015 – 31st August 2016; this proportion decreased with
241 increasing age-bracket (Table 1).

242 **Table 1.** Statistical data on AGE leading to physician consultation and medical prescription as a
243 function of age-group over the September 1st 2015–August 31st 2016 period.

Age group (years)	< 5	5–65	> 65	All ages
p(AGE with prescription)	0.38	0.09	0.04	0.10
$p_{AGE\|prscr.}$(antiemetic only)	0.29	0.43	0.36	0.39
$p_{AGE\|prscr.}$(antidiarrheal only)	0.24	0.17	0.26	0.19
$p_{AGE\|consult.}$(antiemetic and antidiarrheal)	0.20	0.33	0.25	0.30
$p_{AGE\|prscr.}$(ORS <1 year)	0.02	0	0	0.004
$p_{AGE\|prscr.}$(ORS 1-15 years)	0.24	0.002	0	0.05
$p_{AGE\|prscr.}$('Others')	0.02	0.07	0.13	0.07
$p_{AGE\|prscr.}$(vomiting symptom)*	0.67	0.82	0.70	0.79
p_{AGE}(vomiting symptom)**	0.48	0.67	0.51	0.63
p_{AGE}(viral infection)***	0.00	1.00	0.00	0.77

244 *assessed from the sum of p_{AGE}(antiemetic) + p_{AGE}(antiemetic and antidiarrheal) and ignoring the
245 ORS and 'Others' groups; **using Equation (4) for a 1-year period; ***using Equation (3) for a
246 1-year period.

247 Prescribed drugs varied with age group, with ORS prescribed to young children ('< 5') only, and
248 'Others' preferentially prescribed to elders (> 65). However, the ORS and 'Others' groups
249 represent a very small proportion of AGE cases with prescriptions (Table 1). For these reasons,

250 and because antiemetics are contraindicated for young children[44,45], we ignored the two ORS
251 groups and the 'Others' group when estimating the proportion of vomiting symptoms among the
252 AGE cases with prescriptions (Table 1). All the values obtained were higher than the probability
253 of vomiting for viral-etiology AGE $p_{\mathrm{AGE|v}}(\mathrm{vom.})$, which suggests viral infections concerned a
254 large proportion of all age groups. The shift from the sub-population with a prescription for AGE
255 to the entire AGE-sick population led to lower estimates of the proportion of vomiting
256 symptoms, which thus fell below the probability of vomiting for bacterial-etiology AGE
257 $p_{\mathrm{AGE|b}}(\mathrm{vom.})$ for the '< 5' and '>65' age-groups (Table 1). This suggests that most AGE did not
258 result from viral infections in these two groups. By contrast, the probabilities of vomiting for the
259 '5–65' age group among the whole AGE-sick population led to an estimate of 100% of viral
260 cases within this group. As the contraindication of antiemetics for young children and the large-
261 scale use of 'Others' drugs for elders could have led to underestimate viral AGE cases in these
262 age groups, we preferred to work on the entire population, largely predominated by the '5-65'
263 age group, regardless of age group.
264 Seasonal variations in probability of vomiting for the whole sick population were estimated
265 from seasonal variations in the corresponding probability of vomiting within the subset of people
266 with a prescription (Figures S3b-c). From these estimates, we deduced seasonal variations in the
267 probability of viral AGE and the number of viral AGE cases (Figures 1a-b). The numbers of new
268 viral AGE cases varied greatly from one day to the next. This strong variability resulted from the
269 variability in number of daily prescriptions (with deviations between consecutive days reaching
270 178 cases, with the biggest difference generally registered from Sunday to Monday) and from the
271 proportions of vomiting symptom that vary randomly in each of the populations suffering from

272 viral or bacterial AGE (Supporting information). The 7-day sliding average greatly reduces this

273 variability (Figure 1b).

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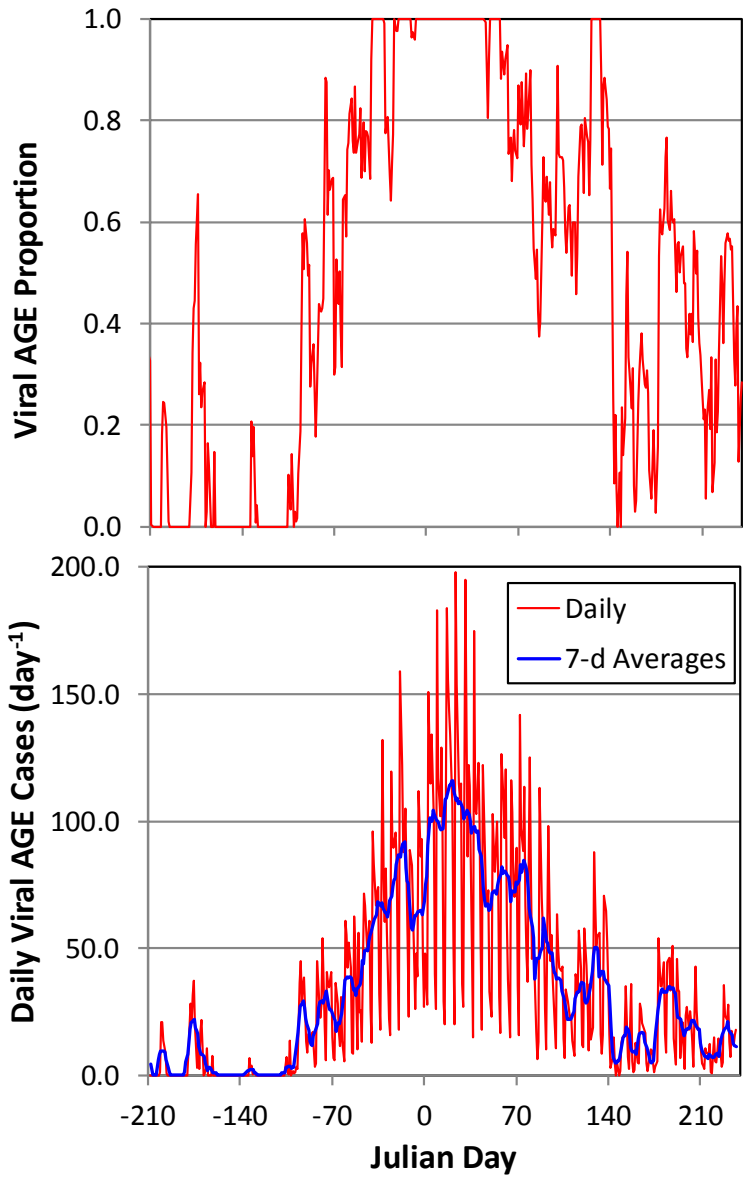

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294 **Figure 1a-b:** Simulation from epidemiological data of (a) proportion of viral AGE, and (b) daily

295 number of viral AGE cases (Day 1: 1st January 2016).

296 Temporal trends in the estimated number of viral AGE cases were consistent with the

297 seasonality of viral AGE[3,50,51]. Only EV (including coxsackievirus and echoviruses) have a

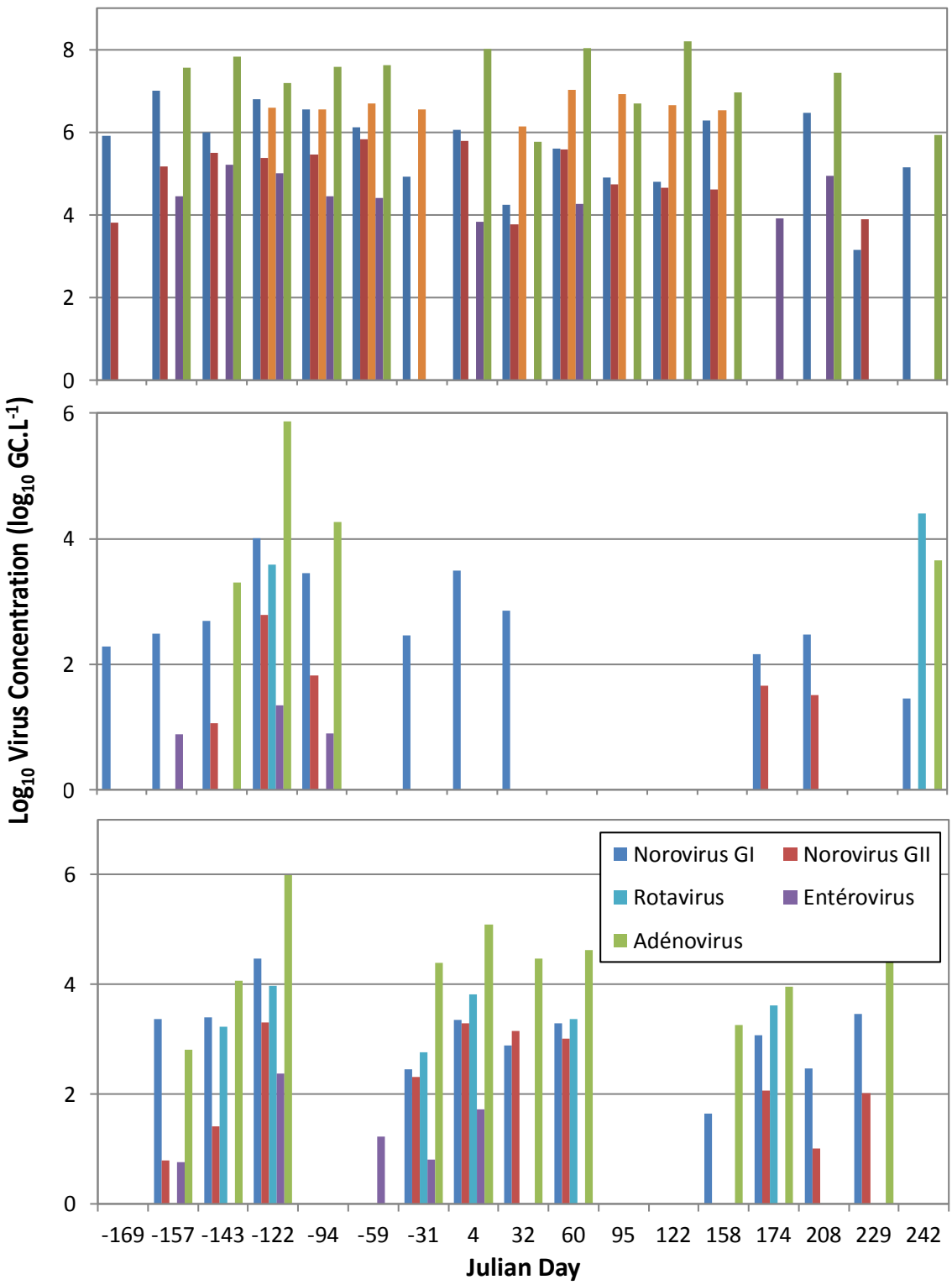
298 summer maximum[52,53]. In Poland, viruses account for 87% and 55% of viral or bacterial
299 gastroenteritis in February and July, respectively, with three times more viral AGE in winter than
300 in summer[50]. Limits to our epidemiological analysis resulted from our working assumptions,
301 including the proportion of vomiting symptoms estimated without taking into account
302 prescriptions of ORS and 'Others', the retained proportions of vomiting symptoms for viral and
303 non-viral AGE cases, and the small size of the population involved.

304 **Viral contaminations of wastewaters and Artière River water: WWTP efficiency.**

305 All quantified viruses were almost systematically found in raw wastewaters, with the exception
306 of HAV which was never detected (Figure 2a). The non-detection of HAV could be explained by
307 the fact that it is rare in most European countries like France except after contaminations due to
308 international exchanges[54]. Note however that HAV had sometimes been detected at the inlet of
309 the same WWTP in another study one year earlier[55]. When above the detection thresholds, the
310 concentrations of other viruses ranged from (in $GC.L^{-1}$) $10^{+3.1}$ to $10^{+7.0}$ for NVGI, $10^{+3.8}$ to $10^{+5.8}$
311 for NVGII, $10^{+5.1}$ to $10^{+7.0}$ for RV-A, $10^{+3.8}$ to $10^{+5.2}$ for EV, and $10^{+5.8}$ to $10^{+10.0}$ for AdV
312 (Figure 2a). These recovery rates were equivalent to those reported in other European studies and
313 reflect wide virus circulation in the community[55–57].

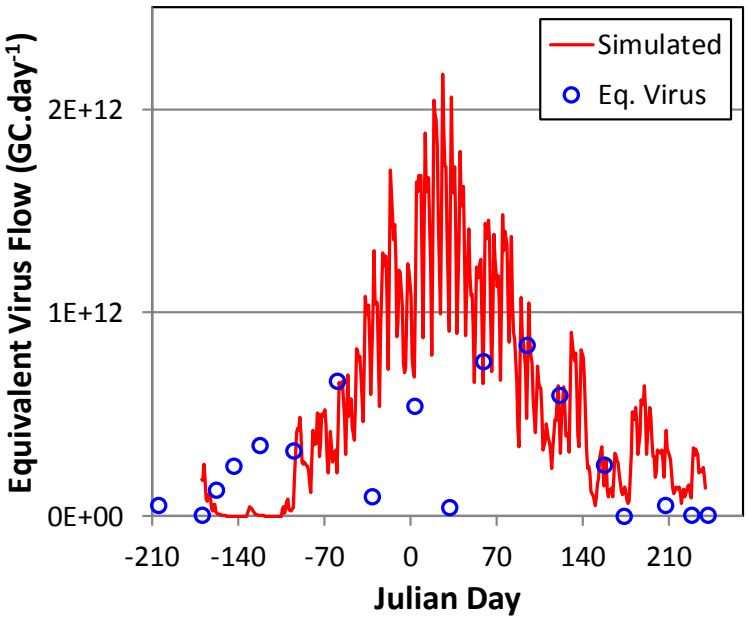
314 When above the detection thresholds, the viral concentrations in treated wastewater at the
315 WWTP outlet (i.e. without additional tertiary treatment in lagoons) were one to a thousand times
316 less than in raw wastewater (results not shown). Detection in WWTP samples was much less
317 sensitive than that for river viruses due to the initial filtration of 1 L of water only. Thus, since
318 treated wastewater discharge contributed between 39 and 90% of the Artière River flow
319 downstream of their point of discharge, the flow of viruses in treated wastewater at the WWTP
320 outlet was better estimated from the increase in the amount of viruses carried by the Artière

321 River between upstream and downstream of the wastewater discharge point plus wastewater
322 withdrawal for filling the lagoons minus virus flow resulting from WWTP by-pass. Virus
323 removal efficiency in the WWTP was then estimated by the ratio of virus flow between inlet and
324 outlet (Figure S4). The annual geometric averages of virus removal within the WWTP over the
325 annual period 1st September 2015 – 31st August 2016 were 2.4, 1.9, 3.3, 2.6 and 2.5 $\log_{10}$ for
326 NVGI, NVGII, RV-A, EV and AdV, respectively. These virus removals were *a priori* higher
327 than those measured in the WWTP of similar treatment trains[33,34,58] , which may be due to the
328 long residence time of wastewater in this treatment plant (2–3 days), the anoxic zone of the
329 activated sludge basins, and the use of iron chlorosulfate to precipitate phosphates during
330 clarification. Even though high variability between estimates led to uncertainties in the seasonal
331 variations (Figure S4), the differences in $\log_{10}$ removals between summer 2015 and winter 2016
332 were 0.9, 2.7, 1.7 and 2.2 for NVGI, NVGII, EV and AdV, respectively.
333 While the Artière River generally only received treated wastewater from a small WWTP
334 (120 population equivalents) upstream of the discharge by the *'Trois Rivières'* WWTP, virus
335 concentrations upstream were sometimes of similar order of magnitude to virus concentrations
336 downstream of the *'Trois Rivières'* WWTP discharge point (Figures 2b-c), as it was possible for
337 wastewater to discharge directly from the sewer system upstream of the *'Trois Rivières'* WWTP.
338 When above the detection thresholds, virus concentrations in the Artière River downstream of
339 the WWTP discharge ranged from (in $GC.L^{-1}$) $10^{+1.6}$ to $10^{+4.1}$ for NVGI, $10^{+0.8}$ to $10^{+3.3}$ for
340 NVGII, $10^{+2.8}$ to $10^{+4.0}$ for RV-A, $10^{+0.8}$ to $10^{+2.4}$ for EV, and $10^{+2.8}$ to $10^{+6.0}$ for AdV (Figure
341 2c). These orders of magnitude were in agreement with published data[2–4], and with the seasonal
342 variations in quantity[59,60].
343



Figures 2a-c: Measured virus concentrations (a) in raw wastewaters, (b) in the Artière River upstream treated wastewater discharge, and (c) in the Artière River downstream of the treated wastewater discharge.

371 **Comparing the simulated shedding and environmental fate of viruses to the**
372 **weighted sum of the virus flows monitored *in situ.*** The simulated quantities of daily
373 virus shedding were compared to the weighted sums of the flows of viruses monitored at the inlet
374 of the WWTP, after having estimated the weighting coefficients a, b, c, d and e for NVGI,
375 NVGII, RV-A, EV and AdV, respectively. There was good agreement between simulated trends
376 and weighted observations, with near-zero flows during summertime and flows reaching about
377 10^{12} GC.day^{-1} during the winter period, despite occasional large deviations in wintertime (Figure
378 3). Note that qPCR measurements have high uncertainties, making their use more appropriate for
379 variations over several powers of 10 than for variations of viral AGE numbers of less than 10
380 between winter and summer.
381



382
383
384 **Figure 3:** Simulated total daily virus shedding based on epidemiological data *versus*
385 experimental equivalent virus concentrations.

386

387 NVGI and EV did not contribute to the equivalent virus concentrations (i.e. a = d = 0), whereas
388 the relative contributions of the other three viruses to the equivalent virus concentrations
389 decreased in the order NVGII ($b = 10^{-2}$) > RV-A ($c = 5.8\ 10^{-4}$) > AdV ($e = 4.6\ 10^{-5}$). The lower
390 concentrations of EV compared to other viruses could explain why d = 0. For the other more
391 abundant viruses, their specific weighting coefficients should *a priori* be proportional to the
392 "number of AGE cases-to-quantity of shed viruses for all carriers" ratio for a given virus. Since
393 some of the monitored viruses may cause AGE symptoms other than the vomiting and/or
394 diarrhea, carriers without AGE symptoms should be distinguished from those with AGE
395 symptoms, rather than distinguishing asymptomatic from symptomatic carriers as often done.
396 Virus concentrations in the Artière River downstream wastewater discharge were simulated by
397 mixing water coming from the Artière River upstream of the wastewater discharge, treated
398 wastewater discharged into the Artière River (i.e. not withdrawn for filling lagoons), and raw
399 wastewater discharged without treatment through the WWTP bypass. For this purpose, virus
400 removal in the WWTP was simulated in each basin zone with $Q_{10} = 1.7$ (Supporting
401 Information). Simulated virus concentrations were compared to equivalent virus concentrations
402 using the weighting coefficient values already estimated from virus flow in raw wastewaters
403 (Figure 4). There was good agreement between simulated trends and weighted observations, with
404 the exception of a very high weighted observation on September 1st, 2015 and two consecutive
405 zero values between early April 2016 and early May 2016 (Figure 4). A bypass of 320 m³ was
406 operated on September 1st, 2015, the day when the weighted observations corresponded to a
407 concentration in significantly higher equivalent viruses ($6\ 10^{+5}$ viruses.L^{-1}) than the value
408 simulated from epidemiological data ($1.9\ 10^{+2}$ viruses.L^{-1}). At this date, it is likely that the by-

pass (very low compared to other dates) was operated during less than 1 hour concomitantly to our water sampling, leading to a higher spot contamination of the Artière River than the simulated value assuming that the by-pass was operated over 24 hours. Two other peaks reaching about 1.5 10^{+6} virus.L^{-1} were simulated using epidemiological data on two other days with a daily by-pass that could reach 10500 m^3.day^{-1} (more than 30 times the by-pass flow of 1st September 2015). Beyond this spot difference, the simulated results show how much operation of the by-pass can occasionally (but recurrently) affect contamination of Artière River (Figure 4).

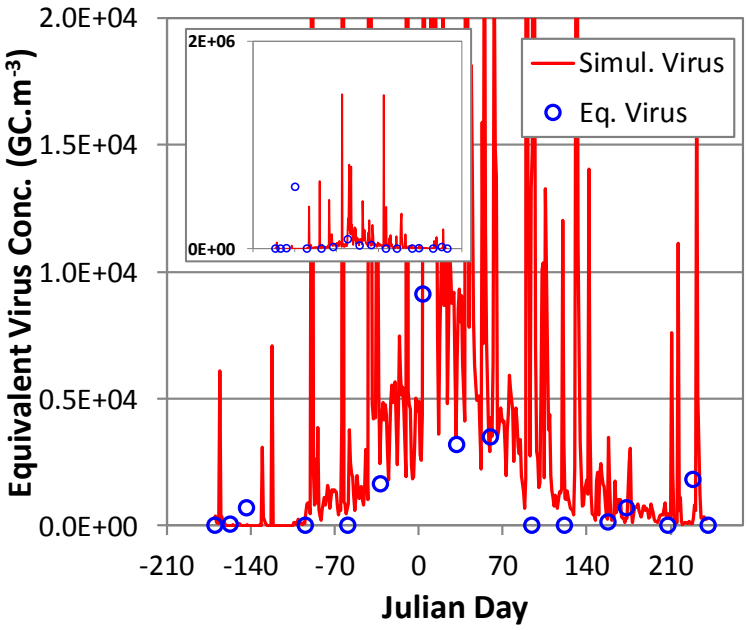


Figure 4: Comparisons of simulated virus concentrations in the Artière River downstream of the wastewater discharge when neglecting virus contamination of the Artière River upstream of the wastewater discharge against the sum of weighted measured virus concentrations. (Virus contamination of the Artière River upstream of wastewater discharge could have increased the simulated equivalent virus concentrations between about 9 10^{+1} and 1 10^{+3} GC.L^{-1}, except on 1st September 2015 where the increase could have reached 7 10^{+4} GC.L^{-1}).

434

435 Thus, our model combining AGE epidemiology, viral excretion and subsequent environmental
436 fate of human pathogenic enteric viruses makes it possible to simulate the main trends observed.
437 This model can thus serve to compare treatment, discharge and reuse scenarios. Limits in the
438 comparison between simulations and weighted experimental data resulted from some of the
439 model-simplifying assumptions (e.g. virus removal neglected in the sewerage system, additional
440 water sources and sinks in the Artière River) and experimental limits, including other AGE-
441 related viruses (e.g. aichiviruses and astroviruses)[3] that were not quantified

442

443 **ASSOCIATED CONTENT**

444 **Supporting Information**.
445 The Supporting Information is available free of charge on the ACS Publications website at DOI:
446 XXXXXX.

447 (1) Sampling zone, (2) Uncertainty about the number of viral AGE number due to
448 variability of the vomiting symptom, (3) Seasonal variations in the probability of
449 vomiting for the whole population, and (4) Virus removals in the *'Trois Rivières'*
450 WWTP; measurements and simulations (PDF)

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647

Supporting information

Vomiting symptom of acute-gastroenteritis estimated from epidemiologic data can help to assess river contamination by human pathogen enteric viruses

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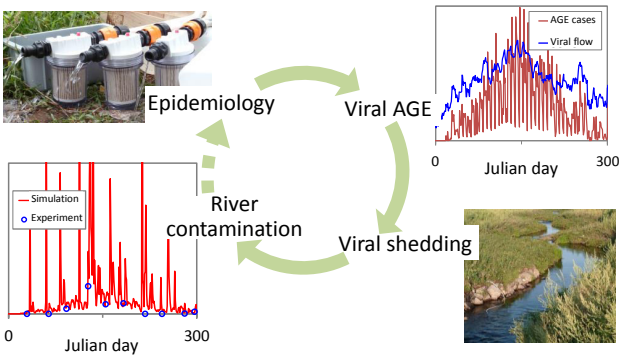
KEYWORDS: Human enteric viruses, Acute gastroenteritis, Epidemiology, Shedding, Wastewater treatment plant, River contamination, Environmental fate, Model.



1. SAMPLING ZONE

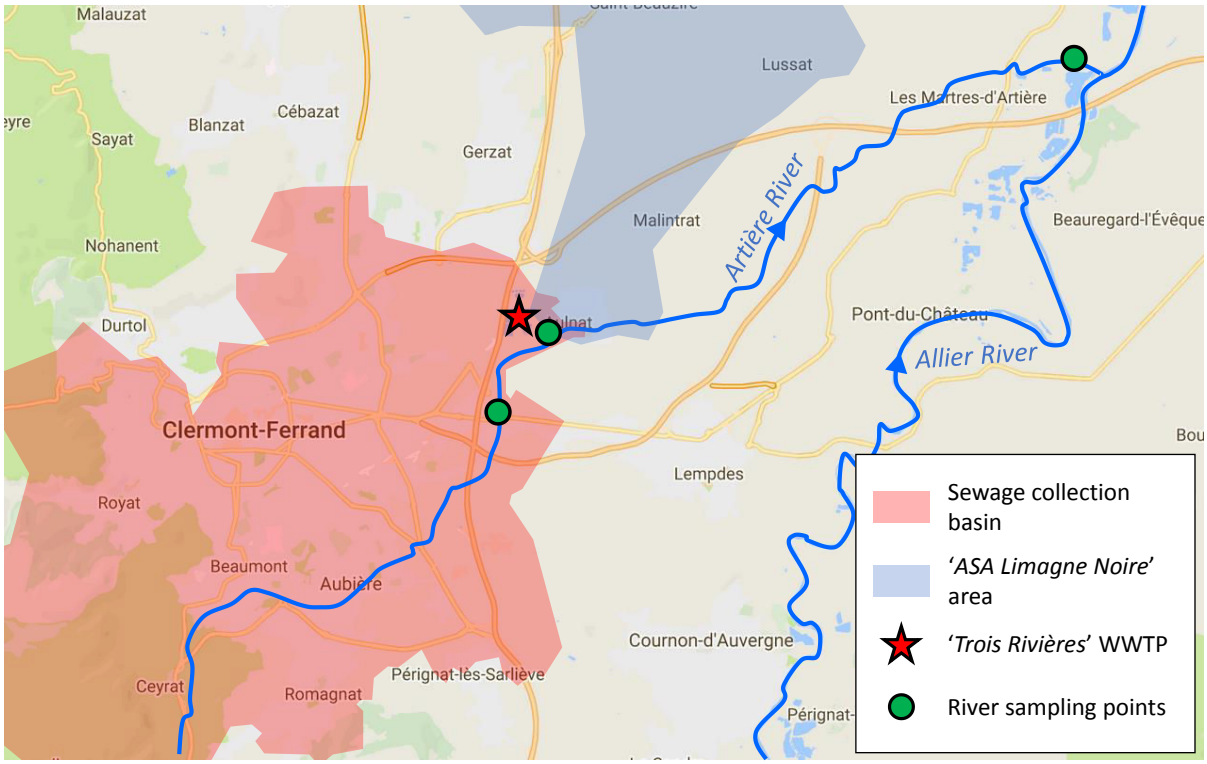
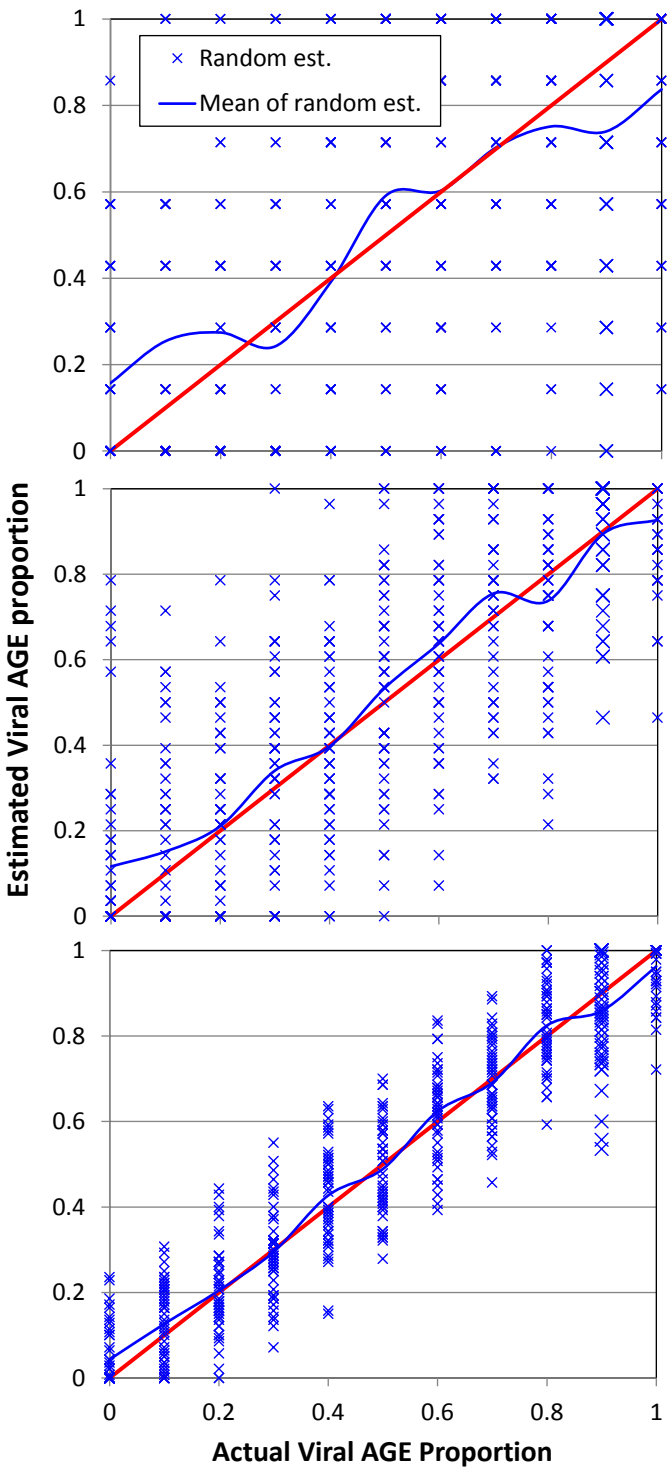


Figure S1: Sampling positions relative to the sewage collection basin, the WWTP and the area managed by *'ASA Limagne Noire'* farmer association and irrigated by treated wastewater.

2. UNCERTAINTY IN THE NUMBER OF VIRAL AGE CASES DUE TO THE VARIABILITY OF VOMITING SYMPTOM

Uncertainties in the number of daily new viral AGE cases resulted from the variability in the number of daily consultations and the proportions of vomiting symptoms in the population suffering from viral or bacterial AGE. We assumed that the probabilities to vomit were 0.52 and 0.66 for each AGE case of bacterial and viral etiologies, respectively. For each of 50, 200 and 1000 people suffering from AGE sets and for each of viral AGE proportions varying from 0 to 1 in steps of 0.1, we performed 50 Monte Carlo simulations (Figures S2).



Figures S2a-c: proportions of viral AGE estimated from the actual viral AGE proportions for Monte Carlo simulations, assuming that vomiting probabilities were 0.52 and 0.66 for viral and bacterial AGE cases, respectively, for (a) 50, (b) 200 and (c) 1000 total AGE cases.

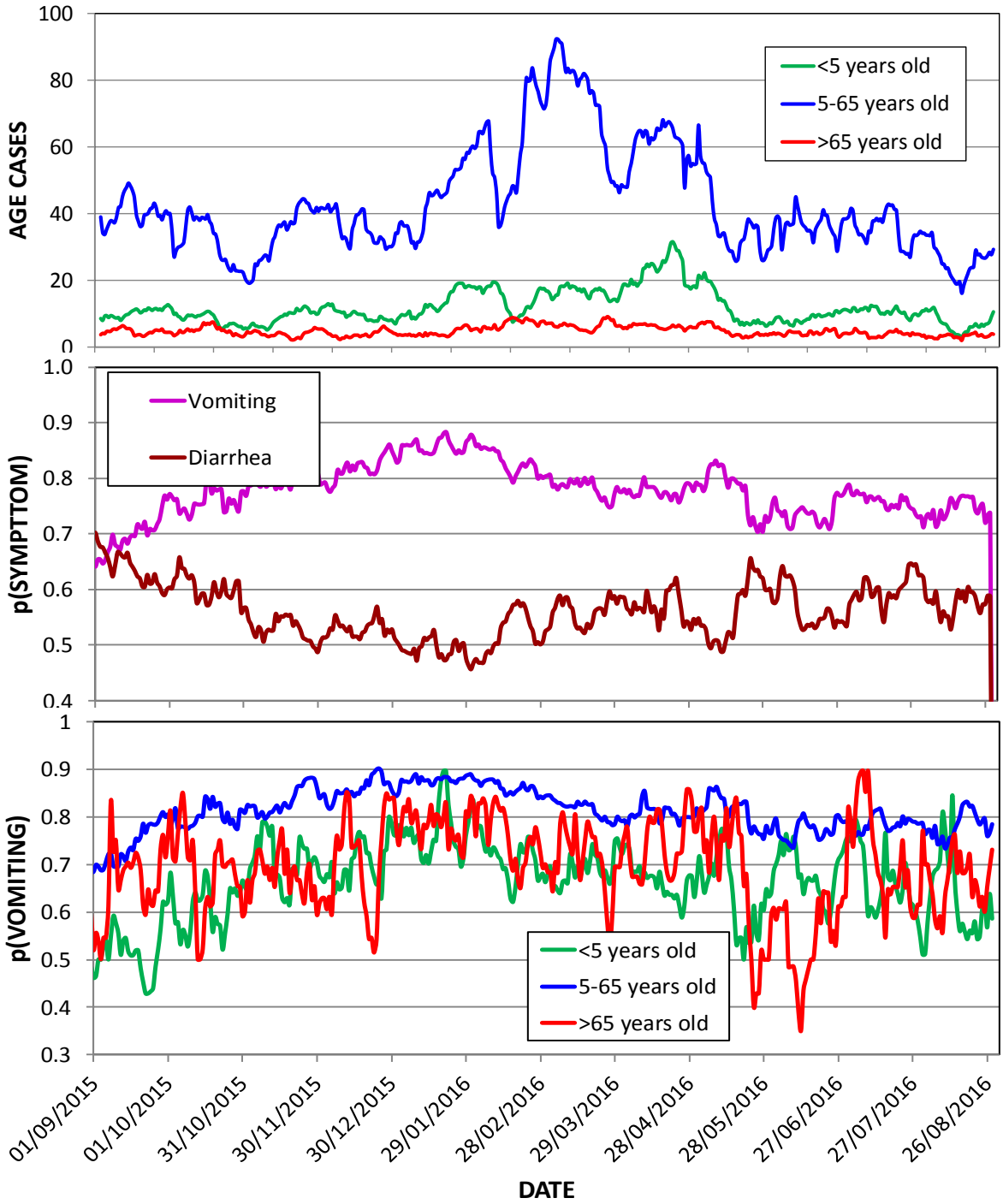
The uncertainty in the resulting estimated proportion of viral AGE decreased with increasing the total number of AGE cases. Overestimations and underestimations of the proportion of viral AGE were noted for actual proportions of viral AGE near 0 and 1, respectively, but they decreased with increasing the total number of AGE cases. The use of sliding averages over 7 days greatly minimized the uncertainties resulting from the random nature of the vomiting symptom (Figure 1b of the research article).

3. SEASONAL VARIATIONS IN THE PROBABILITY OF VOMITING FOR THE WHOLE POPULATION

Seasonal variations in the probability of vomiting for the whole population were estimated from seasonal variations in the probability of vomiting within the subset of sick people consulting a physician (Figures S3a-c). Differences between the three age groups '< 5', '5-65' and '> 65' in AGE or symptoms could help to better identify the potential origins of the AGE. For example, the increase at the end of 2015 in the number of AGE cases in the '5-65' age group probably resulted from the consumption by this group of raw oysters during Christmas time.

4. VIRUS REMOVALS IN THE *'TROIS RIVIÈRES'* WWTP; MEASUREMENTS AND SIMULATIONS

Virus removals (i.e. virus inflow – to – virus outflow ratio or its decimal logarithm) within the WWTP were estimated for each of the quantified viruses at each sampling date. Virus inflows were estimated from measured wastewater flow and virus concentrations within raw wastewaters. Virus outflows could be estimated from either (i) virus concentrations in treated wastewaters, or (ii) the increases in virus flow in the Artière River resulting from treated wastewater discharge, taking into account any by-pass of raw wastewater and/or treated



Figures S3a-c: Seasonal variations in AGE with medical prescriptions. (a) AGE for each age group; (b) probabilities of vomiting and/or having diarrhea for the all age groups; and (c) probabilities of vomiting for each age group. *(Gray areas periods where AGE and/or symptoms varies between age groups)*

wastewater withdrawal for filling lagoons used for crop irrigation. Since virus concentrations were measured from only 1 L treated wastewater, compared with 100 L of water in the Artière River upstream and downstream of wastewater discharges, virus outflows were better estimated from the increases in virus flow in the Artière River, this last option leading to less values below the detection threshold of qPCR. Using the increase in virus flow in the Artière River, virus removals were estimated at each sampling date for each of the quantified viruses (Figure S4).

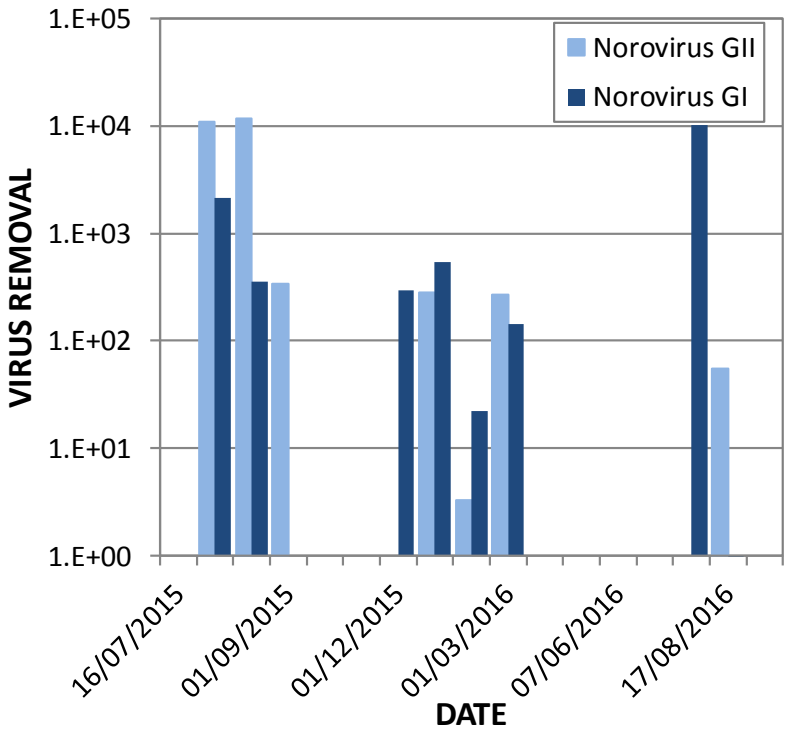


Figure S4: NVGI and NVG2 removals at each sampling date.

The geometric averages of virus removals within the WWTP over the annual period 1st September 2015 – 31st August 2016 were 2.4, 1.9, 3.3, 2.6 and 2.5 $\log_{10}$ for the NVGI, NVGII, RV-A, EV and AdV, respectively. The high variability between estimates led to uncertainties in the seasonal variations; nonetheless, the differences of $\log_{10}$ removals between summer 2015 and winter 2016 were 0.9, 2.7, 1.7 and 2.2 for NVGI, NVGII, EV and AdV, respectively. We checked whether these orders of magnitude could be explained assuming seasonal variations in wastewater flow and temperature (Figure S5).

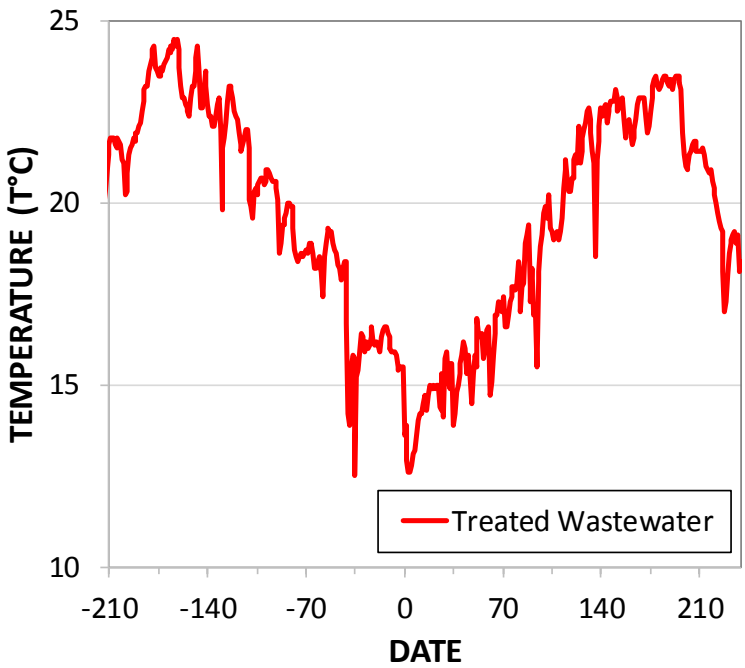


Figure S5: Seasonal variations in wastewater temperature at the outlet of the treatment plant.

The treatment train of the *'Trois Rivières'* WWTP combines a pre-treatment with screening, degreasing and sand sedimentation, an activated sludge treatment with anoxic, hypoxic and aerated zones, and a clarification using iron chlorosulfate to precipitate phosphates and organic polymers to precipitate the activated sludge. Virus removal within the *'Trois Rivières'* WWTP was thus simulated by considering removals successively within the primary settling tank, the anoxic, hypoxic and aerated zones of the activated sludge tanks, and the clarifiers. In each of these compartments, we assumed the following equation:

$$V_{\mathrm{t}} \times \frac{\partial C_{\mathrm{o}}}{\partial t} = (C_{\mathrm{i}} - C_{\mathrm{o}}) \times Q_{\mathrm{w}} - k_{\mathrm{t}}(T) \times V_{\mathrm{t}} \times C_{\mathrm{o}} \qquad \text{(S1)}$$

where V_{t} is the volume of the considered tank (m^3), C_{i} and C_{o} the virus concentrations (GC L^{-1}) at the inlet and outlet of the tank, respectively, t the time (hour), Q_{w} the water flow (m^3 h^{-1}), and k_{t} the tank removal rate (h^{-1}) depending on the temperature T (°C) of wastewater. C_{o} is also the virus concentration within the tank.

We set $k_{\mathrm{t}}(T)$ values at 20°C to 0.1, 0.6, 0.4, 0.4 and 0.1 $hour^{-1}$ for the primary settling tank, the anoxic, hypoxic and aerated zones of the activated sludge tank, and the clarifiers, respectively.

We assumed that variations in the removal rates with temperatures could be described by a Q_{10} equation:

$$k_t(T) = k_t(20°C) \times Q_{10}^{\frac{T-20}{10}} \quad \text{(S2)}$$

where Q_{10} is the proportional increase of k_t with 10°C increase in temperature. We assumed that Q_{10}=1.7. The mean temperature T of all the tanks of the WWTP were assumed equal to the temperature measured in the treated wastewater outflow. For each season, we present in Figure S6 examples over 48 hours periods without by-pass.

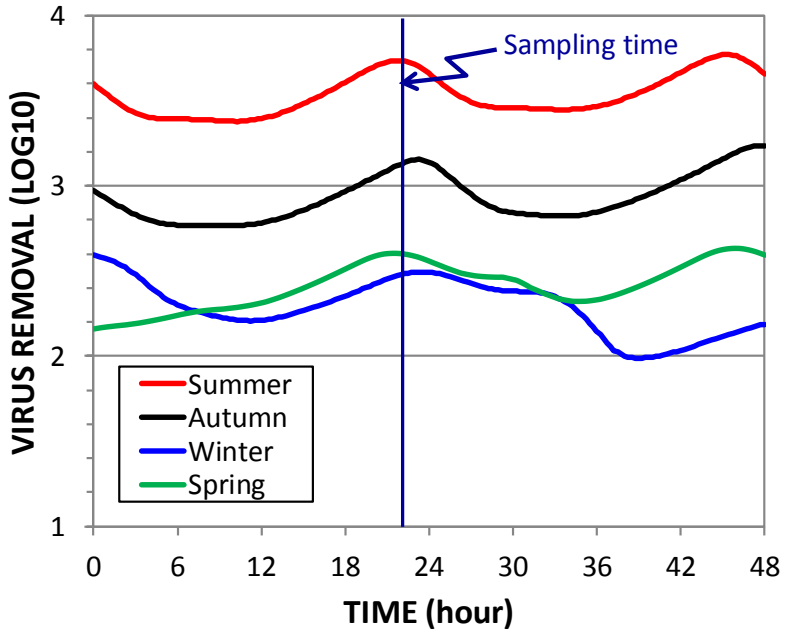


Figure S6: Variations in the apparent virus removal ($\log_{10}$) during four 48 h periods in autumn (November 6-8 2015), winter (January 18-20 2016), spring (April 6-8 2015) and summer (August 11-13 2015).

The daily virus removal within the WWTP over the 1st September 2015 – 31st August 2016 period was simulated by simultaneously using this simplified modelling and taking into account wastewater bypass that explained some days most of Artière viral contamination (**Fig. 4** of the research article).

Apparent virus removal (i.e. virus inflow – to – virus outflow ratio for simultaneous raw and treated wastewater samplings) varied hourly with 0.5 $\log_{10}$ differences between minimum and maximum values (**Fig. S6**). We noted seasonal variations with mean removal rates of 2.9, 2.2, 2.5 and 3.3 for the considered periods in autumn, winter, spring and summer seasons, respectively. In addition for simultaneous raw and treated wastewater samplings at about 10 h a.m. UT, the apparent virus removals exceed the daily averages of about 0.2, 0.3, 0.1 and 0.2 $\log_{10}$ for the considered periods in autumn, winter, spring and summer seasons, respectively.

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Annexe III

Spatial correlations in daily acute gastroenteritis between cities

* Cette Annexe correspond à l'*Appendix 1* de l'article *"An attempt to predict the concentrations in human enteric viruses in the French Artière and Allier rivers from epidemiologic and drug consumption data"*, cosigné par Tesson V., Belliot G., Estienney M., Wurtzer S., Renault P, prochainement soumis à *Environmental Science & Technology*

Appendix 1

Spatial correlations in daily acute gastroenteritis between cities

Although this aspect is not directly related to the objectives of this study, correlations in declared disease cases between cities were assessed and analyzed with regard to the city sizes, the distances between cities, and the centralization of many activities on Clermont-Ferrand (work, studies ...).

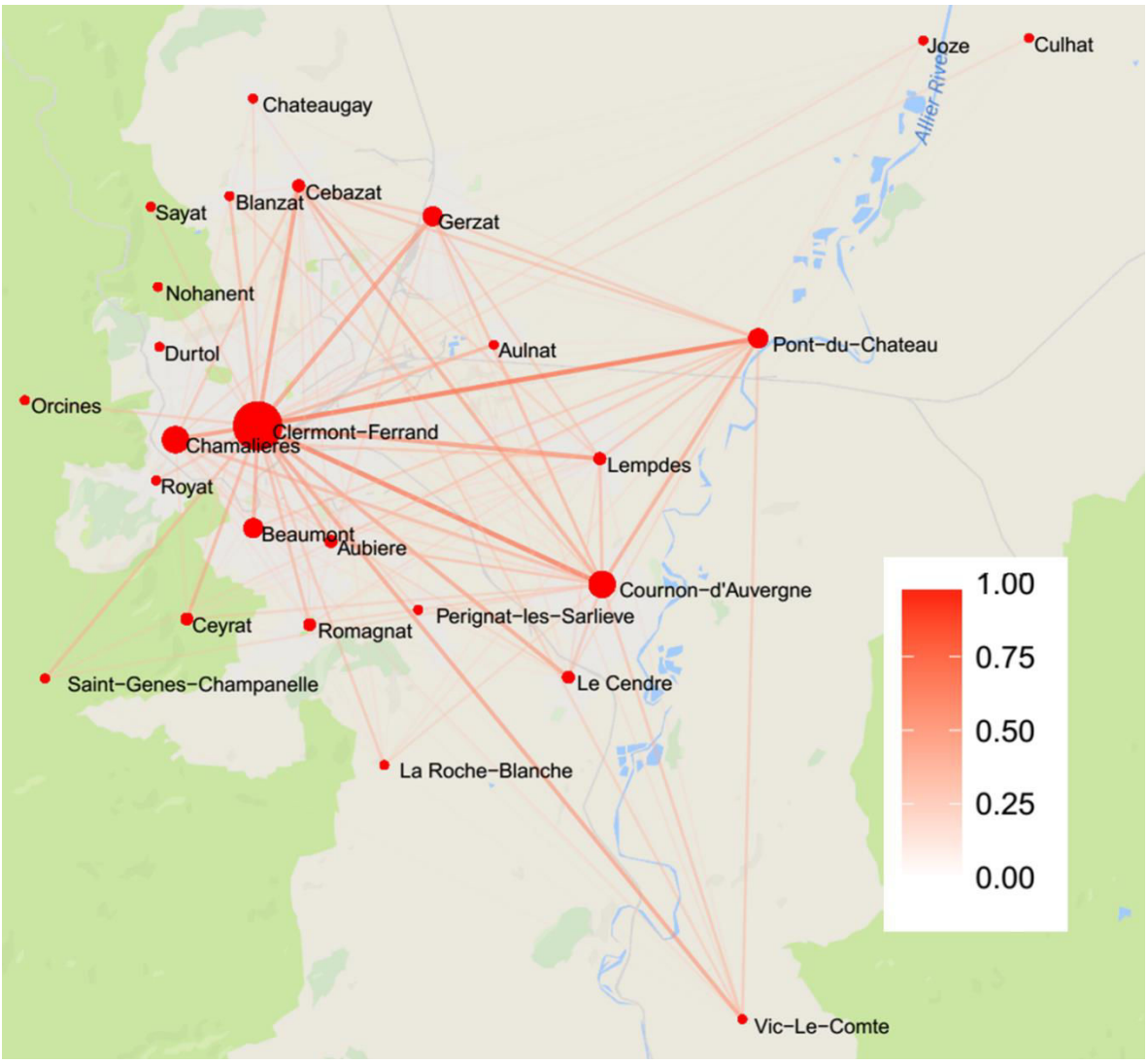

Figure S1.1: Map of correlations between acute gastroenteritis cases according to places of residences

Annexe IV

Modelling the removal and reversible immobilization of murine noroviruses in a phaeozem under various contamination and rinsing conditions

* Cette Annexe correspond au Chapitre III de la présente thèse tel qu'il a été soumis à *European Journal of Soil Science*. Il contient le manuscrit de l'article accompagné des figures et *Supporting Informations*.

Modelling the removal and reversible immobilization of murine noroviruses in a phaeozem under various contamination and rinsing conditions

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Running title: *Modelling the fate of noroviruses in a phaeozem*

Summary

Enteric viruses entering the soil with contaminated irrigation water can reach groundwater or be internalized in plants *via* their roots without being inactivated. Their fate in the soil depends on the virus, the soil and the soil solution. In order to write a mathematical model suitable for a calcaric phaeozem, we investigated murine norovirus removal and reversible immobilization in aggregate columns according to saturation procedure, conditions between times of contamination and rinsing, temperature, and soil solution. Viruses were quantified before and after 0.45 µm filtration using RT-qPCR. Experimental results supported a model combining free and colloidal transport of viruses in mobile water, exchange of free viruses between mobile and immobile waters, virus removal, and reversible virus adsorptions on

suspended colloids, aggregate outer surface and particles within aggregates. For artificial soil solution at 20°C, the fate of viruses in contaminations lasting 1 to 7 days followed by 7 hours rinsing could be described by combining 0.38 $\log_{10}$ daily removal and weak reversible immobilization using a Freundlich isotherm (k_F = 1120, n_F = 1.53), which explained why free viruses prevailed in mobile water. Partial drying without aggregate desaturation did not affect virus recovery. Mg^{2+} enrichment induced geochemical changes that faded over time, resulting in up to ten times more viruses adsorbed on suspended colloids than free, and enhanced adsorption on aggregate outer surfaces; similarly, groundwater slowed remobilization. The fate of murine norovirus within a calcaric phaeozem can be described by a model that takes into account geochemical fluctuations.

Key words: wastewater reuse, enteric virus, soil, virus fate, geochemical changes, simulation

Acknowledgments

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for aggregate bulk density measurements. Lastly, we thank Richard Ryan for English editing and proofreading.

Highlights

- How far does soil reduce the risk that noroviruses brought by irrigation reach groundwater or roots?
- Virus removal over a five-day average residence time in phaeozem is about two $\log_{10}$.
- Reversible virus immobilization is weak; Mg intake favours it, together with colloidal transport.
- Virus removal and reversible adsorption may be modelled to better assess the risk of contamination.

Introduction

Wastewater reuse for crop irrigation can help address increasing water scarcity and declining conventional water quality by upgrading marginal fertilizing resources and curbing their discharge in conventional water bodies (Jaramillo & Restrepo, 2017). However, their chemical and microbial contents imply environmental, agricultural and health risks. Domestic wastewaters generally contain human enteric viruses (Jaramillo & Restrepo, 2017) that have often been identified as causing waterborne and foodborne disease outbreaks in industrialized countries (Kirk *et al.*, 2015). Noroviruses, reportedly responsible for 36% and 67% of gastroenteritis in the world and in Europe, respectively (Kirk *et al.*, 2015), are by far the leading cause of outbreaks associated with the consumption of leafy vegetables in the USA (Herman *et al.*, 2015). Their environmental transmission is favoured by strong excretion over weeks by both sick and healthy carriers (Teunis *et al.*, 2015), resistance to conventional water treatments, persistence in the environment for several weeks to months (Jaramillo & Restrepo, 2017), and low infectious dose (Atmar *et al.*, 2014).

During irrigation by domestic wastewater, human enteric viruses can be dispersed as bioaerosols or deposited at the surface of the soil and plants. Viruses can then disseminate from the soil surface to the aquifer (Murphy *et al.*, 2017) or be internalized in plants *via* their roots, and then disseminate to their edible portions without being inactivated (DiCaprio *et al.*, 2015). In the soil, virus fate combines transport, immobilization and inactivation (Bhattacharjee *et al.*, 2002). These processes are interdependent: viruses are transferred either free or adsorbed on colloids (Syngouna & Chrysikopoulos, 2010), and virus inactivation may be slowed by their immobilization in biofilms (Skraber *et al.*, 2005) or accelerated by their adsorption on metal oxides at solution pH close to neutrality (Zhuang & Jin, 2008).

Virus immobilization depends on the species, strain and conformation of capsid proteins, on soil minerals and organic compounds, and on the soil solution (Dowd *et al.*, 1998; Zhuang & Jin, 2008; Cao *et al.*, 2010; Michen & Graule, 2010; da Silva *et al.*, 2011). When they are small enough, like noroviruses (27 nm), in contrast to bacteriophage PM2 (60 nm), and not aggregated by pH near their isoelectric point (Michen & Graule, 2010), their immobilization results from adsorption rather than trapping in pore constrictions or surface crevices (Dowd *et al.*, 1998). Forces involved in virus adsorption include Van der Waals, electrical, hydrophobic and Born forces (Park & Kim, 2015). Whereas Van der Waals forces are always attractive, whether the electrostatic forces are repulsive or attractive depends on virus and soil particle charges, which vary with the pH and ionic composition of the solution (Cao *et al.*, 2010). A few solids, such as Fe oxides, have high isoelectric points that favour virus adsorption over a wider pH range (Syngouna & Chrysikopoulos, 2010). Adsorption is favoured by the presence of divalent cations (Cao *et al.*, 2010; da Silva *et al.*, 2011). Soluble organic compounds may compete with viruses for adsorption (Cao *et al.*, 2010), while solid organic compounds may increase sites on which viruses may be immobilized (Schijven & Hassanizadeh, 2000). Hydrophobic forces are often assumed to result from Lewis acid-base interactions (van Oss, 1993); they vary with the virus (Dika *et al.*, 2013), and are reported to increase with temperature (Syngouna & Chrysikopoulos, 2010).

Although some models of virus immobilization in soil have been part of more integrative models of virus fate in soil or the vadoze zone, with only cursory experimental validations if any (Bhattacharjee *et al.*, 2002), other models are supported by laboratory experiments on immobilization (Tesson & Renault, 2017). Moving viruses have been assumed to be either exclusively free (Sasidharan *et al.*, 2017) or adsorbed on dispersed colloids (Mayotte *et al.*, 2017); in this last case, inert colloid immobilization was assumed to result mainly from filtration (Yao *et al.*, 1971). Virus immobilization has sometimes been held to be irreversible

(Praetorius *et al.*, 2014), as suggested by the colloid filtration theory (Yao *et al.*, 1971) and the extended DLVO theory if the distance between viruses and soil particles corresponds to the primary minimum of energy (Praetorius *et al.*, 2014). More often, virus immobilization has been held to be reversible (Cao *et al.*, 2010; Sasidharan *et al.*, 2017), and explained either by a distance between virus and soil particle corresponding to the secondary minimum of energy, when this minimum exists (Sadeghi *et al.*, 2013), or by geochemical changes that modify energy profile (Cao *et al.*, 2010). Simultaneous reversible and irreversible immobilizations have also been described by distinguishing adsorption sites (Cao *et al.*, 2010) or simulating the kinetic inactivation of reversibly adsorbed viruses (Tufenkji & Elimelech, 2004). Reversible virus adsorption has been kinetically described by introducing attachment and detachment kinetic coefficients (Sasidharan *et al.*, 2017); other works have described equilibrium between viruses in solution and viruses adsorbed on solids with partition coefficient K_D (Syngouna & Chrysikopoulos, 2010), Freundlich or Langmuir isotherms (Vilker & Burge, 1980). However, to our knowledge, no study has dealt with reversible virus immobilization and/or irreversible removal for real soils containing more than 35% clays (Tesson & Renault, 2017).

We set out to collect new experimental data for a calcaric phaeozem (FAO classification), and write a suitable mathematical model, i.e. considering kinetic exchanges or only equilibrium between suspended and reversibly adsorbed viruses (partition coefficient, Freundlich isotherms or Langmuir isotherms).

Materials and Methods

Virus, soil and artificial soil solution

The cytopathic CW1 strain of murine norovirus (MNV-1), as a surrogate of human enteric noroviruses, was kindly provided by H.W. Virgin (University of Washington, MO, US). Noroviruses were propagated on RAW 264.7 mouse leukemic monocyte macrophages (ATCC® TIB-71™) as previously described (Wobus *et al.*, 2004). Two days after infection, the cells and their culture medium were collected, frozen in liquid nitrogen and thawed three times, and centrifuged (10 minutes at 1000 *g* then 30 minutes at 5900 *g*). The supernatant was transferred to Amicon® Ultra-15 tangential filters (Merck Millipore LTD, Ireland) and centrifuged for 40 minutes at 1000 *g*. The pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) to obtain approximately $10^{13.1}$ genomic copies (GC) ml^{-1}. The concentrated viral suspension was frozen in liquid nitrogen and stored at −80°C, later re-aliquoted into 10 µl ready-to-use aliquots, refrozen in liquid nitrogen and stored again at −80°C until experiments began. For each column experiment, one 10 µl aliquot was thawed at room temperature, directly diluted with 40 ml of artificial soil solution, and homogenized for 30 s. Viruses were quantified by RT-qPCR. Practically, 60 µl of viral RNA was extracted from 140 µl of sample following the manufacturer's instructions (QIAamp® Viral RNA kit (Qiagen)), and immediately purified with One-Step™ PCR Inhibitor Removal (Zymo Research Corp, CA) according to the manufacturer's protocol. RT-qPCR was performed as previously described (Belliot *et al.*, 2008) using a Mx3005P® qPCR machine (Agilent Technologies). For a homogeneous virus suspension, the detection threshold of the RT-qPCR analyzes was about 350 GC ml^{-1}, and the standard deviation of the decimal logarithm of measurement replicates was always lower than 0.1.

The 0–15 cm surface layer of calcaric phaeozem (FAO classification) was sampled in the French Limagne Noire, a small region near Clermont-Ferrand (45.85874 N, 3.20088 E). Average annual rainfall was 566 mm for the period 2010–2016. The field where sampling took place had been irrigated yearly with treated wastewater from the wastewater treatment

plant of *Clermont-Auvergne-Metropole* after an additional lagooning tertiary treatment. It had been cultivated with sugar beet since 2015 and the soil was bare, recently tilled but unsown at sampling on 5 January 2016. The soil was stored until use at room temperature at residual moisture of 6.9% dry weight basis. The properties of the 0–15 cm layer were: 3 g kg^{-1} $CaCO_3$, and after decarbonation, 559 g kg^{-1} clay, 222 g kg^{-1} silt, 216 g kg^{-1} sand, 16 5 g kg^{-1} organic C, 1.58 g kg^{-1} total N, 12.7 mg kg^{-1} N-NH_4^+, 29.9 mg kg^{-1} N-NO_3^-. Soil pH(water) was 8.16. Its cation exchange capacity (NF ISO 23470) was 0.465 mol_c kg^{-1} soil, and the concentrations of exchangeable Ca^{2+}, Mg^{2+}, Na^+, K^+ extracted by cobaltihexamine were 0.364, 0.0625, 0.00625, 0.011 mol_c kg^{-1} soil, respectively. Interstratified clays (illite-smectite and illite-smectite-chlorite) were the dominant clays; their behaviour was affected by complex with organic matter (Bornand *et al.*, 1984). The soil probably contained between 4.5 and 6.1% iron (Bornand *et al.*, 1984); 3–4 mm aggregate bulk density was 2.0 g ml^{-1}.

Artificial soil solution was prepared initially from a mixture of ultrapure water, and the soil sampled one year later (5:1 w/v), mixed for 30 minutes, and centrifuged at 10 000 *g* for 30 minutes to recover supernatant, which was filtered through NanoCeram® filters. Soil solution was then autoclaved, aliquoted in 40 ml tubes and stored at 4°C until use. Just before the experiment, some of the 40 mL aliquots of artificial solutions were optionally enriched with the equivalent of 10 mM $MgCl_2$ or 1.25 mg of fulvic acids (1R105F Nordic Aquatic Fulvic Acid Reference, IHSS). The properties of the artificial soil solution, as well as those for groundwater and wastewater are reported in Table S1 (Supporting Information).

Experimental design and procedure

In order to address the partition of viruses in solution between free viruses and viruses adsorbed on dispersed colloids, the reversible immobilization of viruses on undispersed soil

and the removal of viruses due to their irreversible immobilization or to their destruction, it is interesting to vary the conditions that affect soil colloid dispersion (time elapsed after soil wetting, water chemistry), and electrical and hydrophobic forces involved in the adsorption of viruses on soil particles and at air-water interfaces (water chemistry for electrical forces, temperature for hydrophobic forces). Moreover, comparison of virus fate for different soil structures could help assessing the impact of virus transfer on the observed kinetics.

The experimental design therefore combined experiments on immobilization for batches (soil dispersed in stirred solutions) and columns of soil aggregates, with experiments on the possible remobilizing of viruses previously removed from the solution (Figure S1, Supporting Information). Conditions that were varied between the experiments were the initial column saturation procedure (i.e. under partial vacuum or at atmospheric air pressure), saturation of the column just before or well before contamination of the circulating solution, temperature, soil moisture, and chemical conditions.

For experiments on virus immobilization, columns (inner diameter and height 18 mm and 3.6 mm, respectively; total volume 9.2 ml) were initially filled with about 8 g of 3–4 mm or 4–5 mm aggregates of soil at residual moisture, and saturated with the artificial soil solution either under vacuum (0.01 MPa air) or in air to limit or favour the trapping of air bubbles. It results in about 2.65 ml of solid, 0.93 ml of intra-aggregate pores and 5.62 ml of inter-aggregate pores. The solutions circulated in closed circuits between the columns and reservoirs initially filled with 40 ml of the same artificial soil solution at a flow rate of about 4 ml minute^{-1} using a peristaltic pump (Figure 1 and Figures S2a-b, Supporting Information). Approximately 2 ml of water was contained in the tubes between the soil column and the reservoir. The contamination (at time $t = 0$) followed the initial saturation of the column by about 15 minutes or 17 hours depending on whether the quantity of suspended soil colloids, expected to promote transient virus transfer, was to be maximized or minimized. At about 0,

0.25, 0.5, 1, 2, 5, 7 and 24 hours after the contamination, 1.5 ml of soil solution was sampled in the reservoir; 1 ml was filtered at 0.45 µm, and the filtered and unfiltered sampled solutions were distributed in 140 µl, 200 µl and 160 µl for RT-qPCR, cell culture and precautionary storage, respectively. There was only one replicate per date and experiment.

For virus remobilization, some of the columns previously contaminated were first stored for different times and moisture conditions, and then rinsed by virus-free soil solutions at a flow rate of about 4 ml minute^{-1} by creating an open circuit with the reservoir and the column in series (Figure 1). At about 0, 0.17, 0.33, 0.5, 1, 2, 5, 7 hours, 1.5 ml of soil solution was sampled at the column outlet.

Batch incubations were performed in sealed 50-millilitre tubes initially filled with 4 g of air-dried soil and 22.75 ml of the soil solution to have the same solution-to-soil ratio as in the column experiments. The tubes were continuously shaken in darkness for 6 hours. They were inoculated 120, 90, 60, 30, 15, 8, 4, 2 minutes before batch ending with 5 µl of viral suspension to have the same initial contamination per mass of soil as in the column experiments. One tube without soil was inoculated and immediately centrifuged to obtain a zero time point. The tubes were then centrifuged at 10 000 *g* for 30 minutes, and the supernatant was aliquoted as described above.

At the end of the immobilization experiments and after centrifuging the tubes in the batch experiments, the soil solutions were sampled for subsequent analyses at the *Laboratoire d'Analyses des Sols* (Arras, France): pH, organic C, N-NH_4^+, Ca^{2+}, Mg^{2+}, K^+, Na^+.

Process modelling and experimental data analysis

We neglected the volumes of water in the tubes connecting the reservoirs to the soil columns.

For experiments on virus immobilizations with water flowing in a closed loop between the reservoir and the column of soil aggregates, variations in virus concentration in the reservoir may be written:

$$\frac{\partial(C_{\mathrm{r}}+C_{\mathrm{r-c}})}{\partial t} = \frac{Q}{V_{\mathrm{r}}} \times \left((C_{\mathrm{m}} + C_{\mathrm{m-c}}) - (C_{\mathrm{r}} + C_{\mathrm{r-c}})\right) \quad (1)$$

where t is the time (s), Q the water flow (ml s^{-1}), V_{r} the volume of water in the reservoir (ml), C_{r} and C_{m} the concentrations of free viruses (GC ml^{-1}) in the reservoir and in the mobile water between aggregates within the column, respectively, and $C_{\mathrm{r-c}}$ and $C_{\mathrm{m-c}}$ the concentrations of viruses adsorbed on suspended colloids (GC ml^{-1}) in the reservoir and in the mobile water, respectively.

Virus concentration in the mobile water depends simultaneously on exchange with the reservoir, on exchange of free viruses with intra-aggregate immobile water, and on possible adsorption of free viruses at the aggregate outer surface. Considering that we could neglect colloid immobilization on aggregate outer surface, we assumed that only free viruses can diffuse within the aggregates:

$$\frac{\partial(C_{\mathrm{m}}+C_{\mathrm{m-c}})}{\partial t} = \frac{Q}{V_{\mathrm{m}}} \times \left((C_{\mathrm{r}} + C_{\mathrm{r-c}}) - (C_{\mathrm{m}} + C_{\mathrm{m-c}})\right)$$
$$-\left(\left(\alpha_{\mathrm{e}} \times \frac{1-\varepsilon_{\mathrm{m}}}{\varepsilon_{\mathrm{m}}} \times (C_{\mathrm{m}} - C_{\mathrm{i}})\right) + \left(S_{\mathrm{a}} \times \frac{\partial S_{\mathrm{surf-r}}}{\partial t}\right)\right) \quad (2)$$

where V_{m} is the volume of mobile water between soil aggregates (ml), C_{i} the virus concentration in the immobile water within aggregates (GC ml^{-1}), α_{e} a coefficient for virus exchange between mobile and immobile waters (s^{-1}), ε_{m} the inter-aggregate porosity, S_{a} the aggregate outer surface per unit of mobile water volume (cm^2 ml^{-1}), and $S_{\mathrm{surf-r}}$ the surface concentration of viruses reversibly immobilized on the surface of the aggregates (GC cm^{-2}). For spherical aggregates, α_{e} may be approximated by the following equation (Sardin *et al.*, 1991):

$$\alpha_{\mathrm{e}} \approx 15 \times \frac{D_{\mathrm{v}}}{{r_{\mathrm{a}}}^2} \tag{3}$$

where r_a is the aggregate radius (cm), and D_v the virus (Brownian) diffusion coefficient ($cm^2\ s^{-1}$).

We initially distinguished kinetic reversible immobilization and irreversible removal of free viruses from the immobile water:

$$\frac{\partial C_{\mathrm{i}}}{\partial t} = \frac{\alpha_{\mathrm{e}}}{\varepsilon_{\mathrm{a}}} \times (C_{\mathrm{m}} - C_{\mathrm{i}}) - (k_{\mathrm{ir}} + k_{\mathrm{a-r}}) \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}} \times C_{\mathrm{i}} + k_{\mathrm{d-r}} \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}} \times S_{\mathrm{r}} \tag{4}$$

where ε_a is the intra-aggregate porosity, $\rho_{b\text{-}a}$ the bulk density of aggregates ($g\ ml^{-1}$), S_r the concentration of reversibly adsorbed viruses ($GC\ g^{-1}$), $k_{a\text{-}r}$ the coefficient for reversible virus adsorption on soil solids ($ml\ g^{-1}\ s^{-1}$), k_{ir} the coefficient for irreversible virus removal ($ml\ g^{-1}\ s^{-1}$), and $k_{d\text{-}r}$ the desorption coefficient rate for reversible adsorbed viruses (s^{-1}). We considered both reversible immobilized viruses S_r and removed viruses S_{ir}, respectively ($GC\ g^{-1}$), satisfying:

$$\frac{\partial S_{\mathrm{r}}}{\partial t} = k_{\mathrm{a-r}} \times C_{\mathrm{i}} - k_{\mathrm{d-r}} \times S_{\mathrm{r}} \tag{5}$$

$$\frac{\partial S_{\mathrm{ir}}}{\partial t} = k_{\mathrm{ir}} \times C_{\mathrm{i}} \tag{6}$$

For high $k_{a\text{-}r}$ and $k_{d\text{-}r}$ values and for a constant $k_{a\text{-}r}$-to-$k_{d\text{-}r}$ ratio, C_i and S_r satisfy the following equilibrium:

$$\frac{S_{\mathrm{r}}}{C_{\mathrm{i}}} = \frac{k_{\mathrm{a-r}}}{k_{\mathrm{d-r}}} = k_{\mathrm{D}} \tag{7}$$

where k_D is the partition coefficient ($ml\ g^{-1}$). Equation (4) may then be replaced by the following equation:

$$\frac{\partial C_{\mathrm{i}}}{\partial t} = \frac{1}{\left(1 + k_{\mathrm{D}} \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}}\right)} \times \left\{\frac{\alpha_{\mathrm{e}}}{\varepsilon_{\mathrm{a}}} \times (C_{\mathrm{m}} - C_{\mathrm{i}}) - k_{\mathrm{ir}} \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}} \times C_{\mathrm{i}}\right\} \tag{8}$$

Because Equation (8) requires that adsorbed virus concentration S_r is proportional to solution virus concentration C_i (not valid if adsorption sites differ from each other and/or if the adsorption cannot exceed a maximum), which was not the case in our experiments (results

not shown), other mathematical formalizations have been proposed, including the Freundlich isotherms:

$$S_{\mathrm{r}} = k_{\mathrm{F}} \times C_{\mathrm{i}}^{\frac{1}{n_{\mathrm{F}}}} \tag{9}$$

where n_{F} is a parameter that indirectly describes the deviation from the proportional model involving partition coefficient k_{D}, and k_{F} a proportionality parameter. Virus concentration changes in the immobile water then satisfy the following equation:

$$\frac{\partial C_{\mathrm{i}}}{\partial t} = \frac{1}{\left(1+\left(\frac{k_{\mathrm{F}}}{n_{\mathrm{F}} \times C_{\mathrm{i}}^{\frac{n_{\mathrm{F}}-1}{n_{\mathrm{F}}}}}\right) \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}}\right)} \times \left\{\frac{\alpha_{\mathrm{e}}}{\varepsilon_{\mathrm{a}}} \times (C_{\mathrm{m}} - C_{\mathrm{i}}) - k_{\mathrm{ir}} \times \frac{\rho_{\mathrm{b-a}}}{\varepsilon_{\mathrm{a}}} \times C_{\mathrm{i}}\right\} \tag{10}$$

For experiments on virus re-mobilizations with a flow of water in an open circuit between a large reservoir and the column of soil aggregates, we have:

$$C_{\mathrm{r}} = 0 \tag{11}$$

Equations (2)–(10) may then be used with no other changes. However, the experimentally measured quantity is the concentration of viruses in the water at the outlet of the column.

As immobilization experiments over a one-day period showed no significant trends due to the low adsorption properties of the soil and the high uncertainties of molecular quantifications, except for artificial soil solution enriched with $MgCl_2$, most of the parameter estimations came immobilization experiments over a seven-day period and from subsequent remobilization experiments. As experimental results showed that viruses adsorbed on suspended colloids could be neglected relative to free suspended viruses except when $MgCl_2$ was added to the solution in the reservoir, $C_{\text{r-c}}$ and $C_{\text{m-c}}$ were neglected in the corresponding experiments; in addition, $S_{\text{surf-r}}$ could be neglected in the same experiments. ε_{m} and ε_{a} were estimated assuming a solid density of soil particles and a bulk density of soil aggregates of 2.7 and 2.0 g cm^{-3}, respectively. A single value of the coefficient of exchange α_{e} was empirically estimated for all from 3–4 mm remobilization experiments under saturated conditions to

ensure no apparent discrepancy between experiments and simulations in the time delimiting the transition between initially sharp and later gentle decrease in virus concentration at the column outlet. A single initial concentration $C_r(t = 0)$ and a single set of parameters k_F, n_F, and k_{ir} were then simultaneously estimated by fitting simulations to experimental data for experiments on immobilizations and for experiments on virus remobilizations for 3–4 mm aggregates at 20°C exposed to artificial soil solution without enrichment in $MgCl_2$ or fulvic acids. By contrast, $C_{r\text{-}c}$, $C_{m\text{-}c}$ and $S_{surf\text{-}r}$ were estimated for experiments with $MgCl_2$ enrichment; we then assumed that $C_{r\text{-}c}$ and $C_{m\text{-}c}$ could be described by partition coefficients, while $S_{surf\text{-}r}$ kinetically varied due to micrometre scale transfer:

$$C_{\mathrm{r-c}} = k_{\mathrm{D}} \times \frac{m_{\mathrm{r-c}}}{V_{\mathrm{r}}} \times C_{\mathrm{r}} = k_1 \times C_{\mathrm{r}} \tag{12}$$

$$C_{\mathrm{m-c}} = k_{\mathrm{D}} \times \frac{m_{\mathrm{m-c}}}{V_{\mathrm{m}}} \times C_{\mathrm{m}} = k_2 \times C_{\mathrm{m}} \tag{13}$$

$$\text{with } k_1 = k_2$$

$$\frac{\partial S_{\mathrm{surf-r}}}{\partial t} = k_{\mathrm{c}} \times \left((k_{\mathrm{D}}{}' \times C_{\mathrm{m}}) - S_{\mathrm{surf-r}} \right) \tag{14}$$

where $m_{r\text{-}c}$ and $m_{m\text{-}c}$ are the quantities of colloids in the reservoir and mobile water (g), respectively, k_1 and k_2 adimensional equilibrium constants, k_D' a partition coefficient (ml cm^{-2}), and k_c is a kinetic constant (s^{-1}). As no significant difference was observed for remobilization after the partial drying of soil aggregates, the model was not used specifically for these experiments. For experiments on immobilization after the addition of $MgCl_2$, parameters k_1, k_2 and k_3 were assumed to vary with Mg^{2+} concentration. Finally, the model was fitted to remobilization experiments when groundwater and autoclaved wastewater moved through the column.

Results and Discussion

The mean of the decimal logarithm of the initial virus concentration in the reservoir (8.4) was lower than the value calculated from the dilution of 10 µl of the initial virus suspension into 40 ml (9.5), and their standard deviation (0.5) was higher than the standard deviation resulting from RT-qPCR uncertainty (< 0.1). These observations could result from possible virus removals at the air-water-solid triple interface of 10 µl aliquots before freezing and after thawing, since the triple interface length – to – volume ratio of such aliquots was high (Thompson et al., 1998).

Impact of temperature, aggregate size, soil saturation procedure and water filtration on virus concentrations

The means (and standard deviations) of the differences between decimal logarithms of virus concentrations between 0.45 µm-filtered and unfiltered samples in immobilization experiments were 0.10 (± 0.16), −0.34 (± 0.20) and 0.05 (± 0.25) $\log_{10}$ GC ml^{-1} for soil columns saturated with artificial soil solution 17 hours before contamination, soil columns saturated with artificial soil solution 15 minutes before contamination, and soil columns saturated with artificial soil solution enriched in fulvic acid, respectively. This suggested that virus adsorbed on colloids dispersed in the moving solution could be neglected relative to free viruses. By contrast, the addition of $MgCl_2$ led to a mean difference of 0.71 (± 0.90) $\log_{10}$ GC ml^{-1} between filtered and unfiltered soil solutions.

Experimental conditions gave negligible virus immobilization over 24 hours, and so did not allow aggregate size effect be highlighted by comparing results for batch, 3–4 mm aggregate column, and 4–5 mm aggregate column. In addition, the coefficient for virus exchange α_e estimated by fitting simulations to experimental data for virus immobilization over 1 week and virus subsequent remobilizations over 7 hours suggest that real aggregate

sizes were about one tenth their initial sizes. This agrees with the poor soil structural stability during its wetting, well known for phaeozems, which probably results from the covering of clay particles by humic organic compounds (Bornand *et al.*, 1984).

The impacts of temperature and saturation procedures (under vacuum conditions or under atmospheric air pressure) were also assessed, but were found non-significant (data not shown).

Reversible immobilization and irreversible removal in saturated conditions over a week

For batch and column experiments on virus immobilization, we observed no decrease in virus concentrations over the first 24 hours when the mobile solution was artificial soil solution without enrichment in $MgCl_2$ or fulvic acids (data not shown). The reductions in virus concentrations were presumably too small compared with the uncertainty of molecular quantifications and possible heterogeneities of virus concentrations in the solution.

By contrast, column experiments over 1 week led to a reduction of about 3 $\log_{10}$ in virus concentrations (Figure 2a). The viruses that could be remobilized later with artificial soil solution represented a small fraction of the viruses that previously disappeared in the soil column after sampling and removal of the reservoir (Figure 2b). The quantities of viruses eluted with beef extract from soil columns after remobilizations were barely equal to the quantities of reversibly immobilized viruses that should have been recovered beyond the 7 hours of remobilization (Figure S3, Supporting Information). Non-remobilized viruses may have been irreversibly adsorbed and/or destroyed.

Remobilizations for the samples first subjected to 1, 2, 3 and 7 days of immobilization could be divided into two periods (Figure 2b): (i) the first half-hour during which virus

concentrations at the outlet of the columns $C_o(t)$ (GC ml^{-1}) dropped rapidly in a non-exponential time course, and (ii) the period beyond that, during which virus concentrations decreased slowly in an approximately exponential pattern:

$$C_o(t) \approx c_1 \times 10^{-c_2 \times t} \quad \text{for} \quad t \geq 0.5\ h \tag{15}$$

where c_1 and c_2 are constants specific to each remobilization experiment. c_1 and c_2 were estimated from linear regressions between $\log_{10}(C_o(t))$ and t, and the quantities of viruses that should have been released after 0.5 hour $\hat{Q}_r(t \geq 0.5)$ were approximated by the equation:

$$\hat{Q}_r(t \geq 0.5) = \frac{1}{\ln(10) \times c_2} \times \frac{Q}{m_s} \times c_1 \times 10^{-c_2 \times 0.5} \tag{16}$$

$\hat{Q}_r(t \geq 0.5)$ varied with the final $C_r(t)$ before the beginning of remobilization (Figure 3) according to the following equation:

$$\hat{Q}_r(t \geq 0.5) = 8520 \times \left(C_r(t)\right)^{\frac{1}{1.9}} \tag{17}$$

Extending Equation (15) to the first half-hour (i.e. $\frac{1}{c_2} \times \frac{Q}{m_s} \times c_1$) would have meant multiplying the calculated quantities by 1.08 to 1.38 (results not shown).

The lack of proportionality between $\hat{Q}_r(t \geq 0.5)$ and $C_r(t = 0)$ suggests that immobilization could be described using Freundlich isotherm (Equation (9)), rather than using models based on a partition distribution coefficient k_D (Equation (7)) or kinetic immobilization and remobilization according to Equations (4) and (5a). We described the reversible immobilization of viruses using with $n_F = 1.53$ and $k_F = 1120$ to fit simultaneously virus immobilization and remobilization experiments (Figure 2a, b). Differences between Equation (17) and Freundlich isotherm with these last estimates of n_F and k_F probably result partly from adsorbed viruses being released during the first half-hour in remobilization experiments. For the estimated k_F and n_F values, the soil could retain approximately $10^{3.70}$ GC

g^{-1} soil, $10^{5.66}$ GC g^{-1} soil and $10^{8.93}$ GC g^{-1}, for virus concentrations of 10^1 GC ml^{-1}, 10^4 GC ml^{-1} and 10^9 GC ml^{-1}, respectively.

Regarding the value of $k_{ir} = 1.23 \times 10^{-4}$ ml g^{-1} s^{-1}, it appears that our soil was able to definitively immobilize or destroy 90% of the viruses in solution in approximatively 56 hours in our experimental conditions. We did not observe any impact of the "air-water-poly-propylene" interface on murine norovirus concentration (results not shown) as observed by Thompson *et al.* (1998) for MS2 bacteriophage.

For our phaeozem containing nearly 60% clay and exposed to a concentration of 10^4 GC ml^{-1} of free viruses in solution, the estimated concentration of reversibly immobilized viruses S_r (4.6×10^5 GC g^{-1}) was median with regard to values estimated for various other real soils containing 5–35% clay (Tesson & Renault, 2017); for these last soils, minimum and maximum adsorbed concentrations were approximately between one hundredth and one hundred times more than our estimate (Tesson & Renault, 2017). Although virus adsorption may increase with the cation exchange capacity of soils (Lipson & Stotzky, 1983), other factors affect it, including the nature and quantities of cations in solution, and the presence of metal oxides (Cao *et al.*, 2010; Syngouna & Chrysikopoulos, 2010). A 0.38 $\log_{10}$ irreversible daily removal of viruses was estimated for the phaeozem. It may have resulted from irreversible immobilization, but the fact that no virus was recovered after an attempt to elute them from the soil with beef extract suggests their destruction. This process has most often been ignored in immobilization studies, suggesting that it may be neglected at time scales of immobilization experiments. However, some data on irreversible removal rates have been published: the immobilization rates then ranged between 0.001 and 864 day^{-1} (Tesson & Renault, 2017).

Impact of soil drying on the reversibility of immobilizations

After 24 hours of virus immobilization in column experiments saturated with contaminated artificial soil solution, and partial drying to soil moisture 0.17 g g^{-1}, 0.24 g g^{-1} and 0.36 g g^{-1} dry weight basis following 1–2 days of desiccation, the columns were gently saturated again with virus-free artificial soil solution to prevent the break-up of soil aggregates. Remobilization experiments were then carried out with virus-free soil solution (Figure S4, Supporting Information). The observed concentration differences between remobilization experiments on columns submitted to partial desiccation or not could also be observed on virus initial concentrations in the reservoirs before contamination, so may be due to lower virus concentration in some aliquots. The final state of viruses at the end of the desiccation periods closely depends on the final soil moisture and aggregate bulk density. With an aggregate bulk density $\rho_{b\text{-}a}$ of about 2.0 g ml^{-1} for this soil, the aggregates were never desaturated, even for the lowest experimental soil moisture. This could explain the lack of a drying effect, as water suction probably remained lower than about 2 MPa, ensuring that all the pores of diameter greater than 150 nm remained water-saturated (Fiès, 1992).

Impact of geochemistry on immobilization and subsequent desorption

Virus immobilization was assessed according to several different geochemical characteristics of the circulating solution, with artificial soil solution alone or enriched by either 10 mM $MgCl_2$ or 1.25 mg of fulvic acids. While no significant effect of adding fulvic acids was observed, the addition of $MgCl_2$ led to an important immobilization of viral particles, with a rapid decrease in about 3 hours of the concentrations of viruses in unfiltered samples of more than 2 $\log_{10}$ GC ml^{-1} and concentrations of viruses in filtered samples being approximately one tenth of the concentrations in unfiltered samples, except at the last time (Figure 4). After

the first 3 hours of immobilization, the virus concentrations increased and tended toward values probably equal to about one-tenth of the initial concentration, probably with a narrowing of the differences between filtered and unfiltered samples; unfortunately, we could not determine whether the final concentrations of filtered and unfiltered samples were equal. Viruses in filtered samples were considered free, while viruses immobilized on the filters were considered adsorbed on dispersed colloids. As partial exchange of Mg^{2+} in solution with cations retained on soil cation exchange sites (mainly Ca^{2+}) led to 3.1 mM Mg^{2+} final concentration, we assumed an effect of [Mg^{2+}] on constants k_1, k_2 and k_D' (Supporting Information). In doing so, we obtained simulated values close to experimental ones during the first 3 hours (Figure 4); beyond this time, the re-increase in free virus concentrations was approximately simulated, while the re-increase in the concentration of viruses adsorbed on dispersed colloids was greatly underestimated. This last difference probably results from simultaneous variations in dispersed colloid concentrations that are not described in the model. Moreover, k_F value did not affect virus immobilization in those conditions, suggesting that virus immobilization within soil aggregates could be neglected, compared to virus immobilization on aggregate outer surface and dispersed colloids.

Remobilization experiments were also conducted on aggregate soil columns previously exposed to 24 hours of contamination at 20°C and contaminated artificial soil solution. Taking into account the likely differences between columns for remobilizations with on artificial soil solution, and with either groundwater ($-1.3 \log_{10}$ GC ml^{-1}) or autoclaved wastewater ($-0.35 \log_{10}$ GC ml^{-1}), we did not observe any large differences between the column exposed to virus-free artificial soil solution and the one exposed to autoclaved wastewater (Figure S4, Supporting Information), probably because of the simultaneous higher concentrations in soluble organic matter and mineral cations in wastewater, the effects of which have counteracted each other. By contrast, remobilization of viruses for the

contaminated column exposed to groundwater gave lower remobilization, probably resulting from the groundwater higher Mg^{2+} concentration (Table S1, Supporting Information).

Conclusions and perspectives

Our aim was to collect new experimental data for a calcaric phaeozem and write a mathematical model to describe reversible virus immobilization and irreversible removal (due to irreversible immobilization and/or virus degradation). To our knowledge, our study is the first one on a real soil containing more than 35% clay (Tesson & Renault, 2017). The clay content and its nature, together with the organic matter content, give this phaeozem a high cation exchange capacity; while this value is often positively correlated to virus immobilization, our phaeozem retained few murine noroviruses, possibly owing to complex association between smectite and organic matter, well known in Limagne Noire soils (Bornand *et al.*, 1984). In addition, the high daily virus removal in this phaeozem could have resulted from the simultaneous presence of iron oxides and a solution pH just above neutrality (Zhuang & Jin, 2008).

It was possible to simulate virus fate in this phaeozem with a model combining free and colloidal transport of viruses in mobile water, exchange of free viruses between mobile and immobile waters, kinetic virus removal, and reversible virus adsorptions on suspended colloids and aggregate surfaces, and within aggregates. For experiments without added Mg^{2+}, colloidal transport of viruses could be neglected owing to their low immobilization on soil. By contrast, Mg^{2+} enrichment greatly increased virus adsorption, and so colloidal transport of viruses had to be taken into account, together with virus adsorption on aggregate outer surfaces, outreaching the simple model of exchange between mobile and immobile water.

Practically, a first approximation of the virus average residence time in this phaeozem is probably more than 8 days, corresponding to 3 $\log_{10}$ virus removal. However, viruses brought to the soil quickly disperse in the water, especially in its mobile fraction, which may then be easily absorbed by plant roots.

Supporting informations

The following supporting information is available in the online version of this article. It includes a brief description of additional equations used for describing virus fate for solutions enriched in $MgCl_2$ and the following Table and Figures:

Table S1. Composition of circulating solutions.

Figure S1. Experimental design of the study. GW: groundwater, TWW: treated wastewater, FA: fulvic acids.

Figures S2. Photos of (a) the experimental setup used for monitoring virus immobilization, (b) details of a closed column filled with soil aggregates, and (c) details of an open column.

Figure S3. Final quantities of viruses recovered from the sampled soil solution (Qf samples), the reservoir (Qf reservoir) and the soil aggregate columns (Qf column), compared with initial virus quantity in the reservoir (Q0).

Figure S4. Experimental data for 7 hours virus remobilization experiments after a partial desaturation of the soil columns for 1 day (column DS1d with final $\omega = 0.36$ g g^{-1}) or 2 days (columns DS2d-a with final $\omega = 0.24$ g g^{-1} and column DS2d-b with final $\omega = 0.17$ g g^{-1}). (Simulations were performed using $C_r(t = 0)$, k_{ir}, k_F and n_F estimated for immobilization and remobilization in columns always saturated (see values in caption of Figure 3a, b)).

Figure S5. Experimental virus remobilization experiments for circulating artificial soil solution, autoclaved wastewater, and groundwater, after a first virus immobilization for soil aggregate columns at 20°C and exposed to contaminated artificial soil solution.

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Figure caption

Figure 1 (a) Experimental setup used for monitoring virus immobilization (red flowpath) and virus remobilization (green flowpath), and (b) detail of the components of the column and their assembly.

Figure 2 Experimental data, and simulations assuming $C_r(t = 0) = 1.82 \times 10^6$ GC ml^{-1}, irreversible virus immobilization with $\alpha_e = 2.18 \times 10^{-4}$ s^{-1}, $k_i = 1.23 \times 10^{-4}$ ml g^{-1} s^{-1} and reversible immobilization satisfying Freundlich isotherm with $k_F = 1120$ and $n_F = 1.53$ for S_r in GC g^{-1}. (a) one-week virus immobilization for artificial soil solution as circulating solution; (b) 7 hours remobilizations after 1, 2, 3 and 7 day immobilizations.

Figure 3 Estimated quantities of viruses released at the outlet of soil columns after the first half-hour of remobilization experiments with artificial soil solution, as a function of the final virus concentration during preliminary immobilization. Red dot shows the impact of removing one outlier value not taken into account to characterize the Freundlich isotherm.

Figure 4 Experimental data for 24 hours virus immobilization experiments for circulating artificial soil solution initially enriched with 10 mM of $MgCl_2$. Simulations were performed assuming [Mg^{2+}] and k_1, k_2, k_3 and k_c variations with time (see Supporting Information).

Figure 1

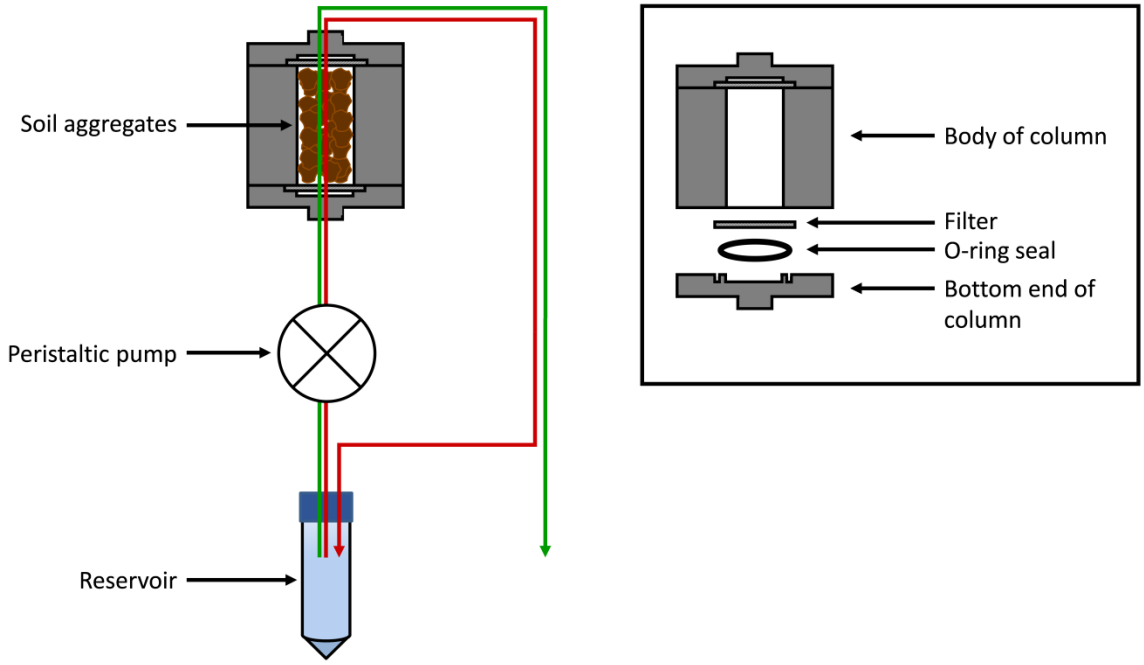
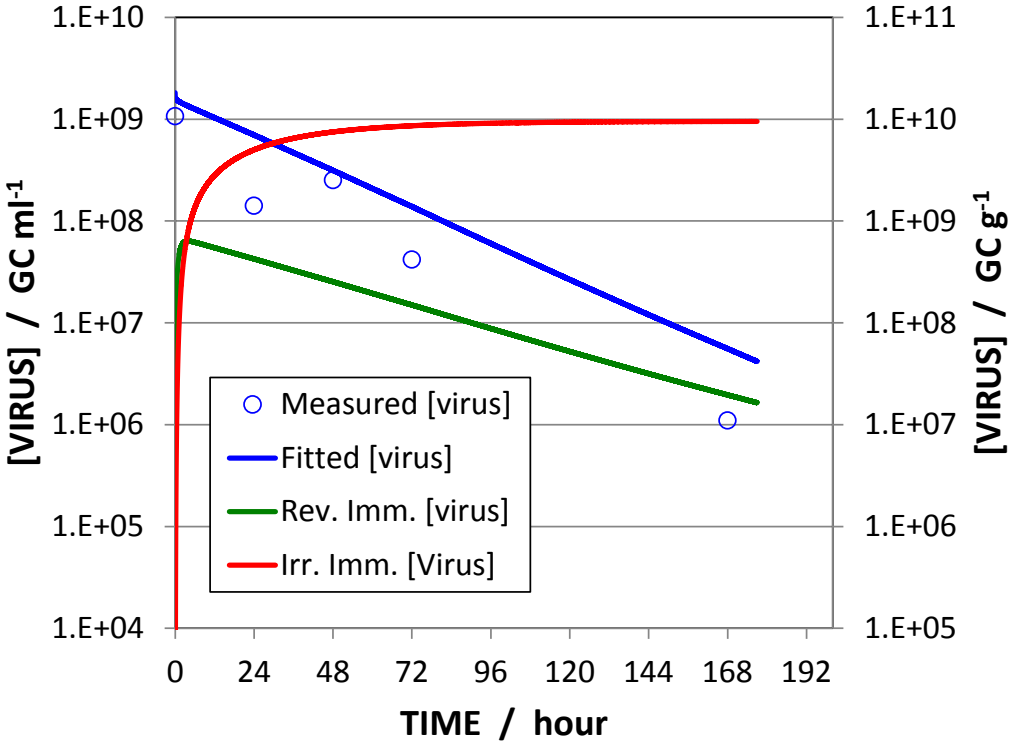

Soil aggregates
Peristaltic pump
Reservoir
Body of column
Filter
O-ring seal
Bottom end of column

Figure 2 a, b


1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
[VIRUS] / GC ml-1
[VIRUS] / GC g-1
0
24
48
72
96
120
144
168
192
TIME / hour
Measured [virus]
Fitted [virus]
Rev. Imm. [virus]
Irr. Imm. [Virus]

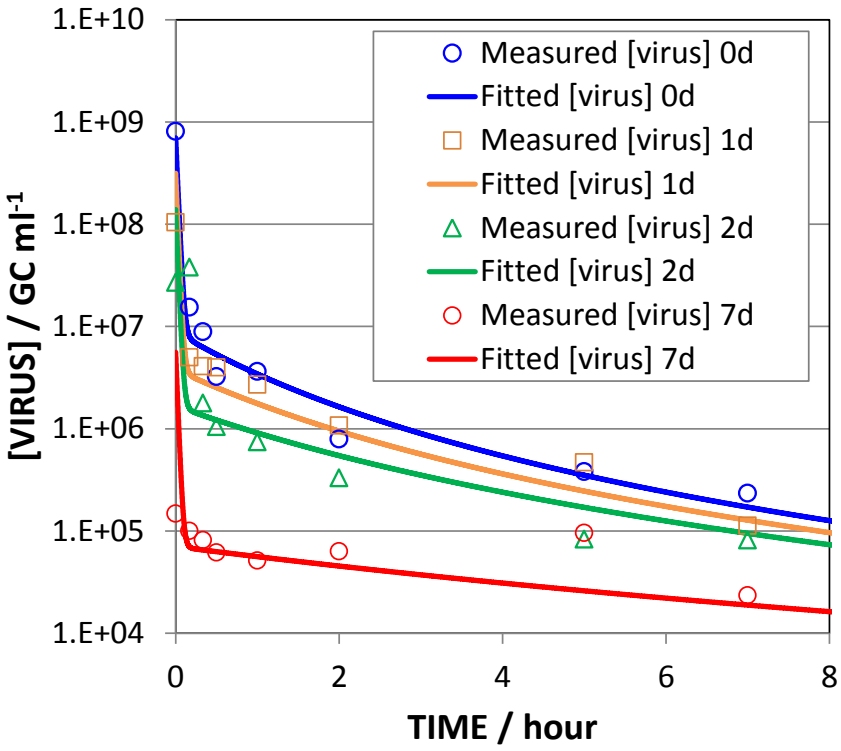
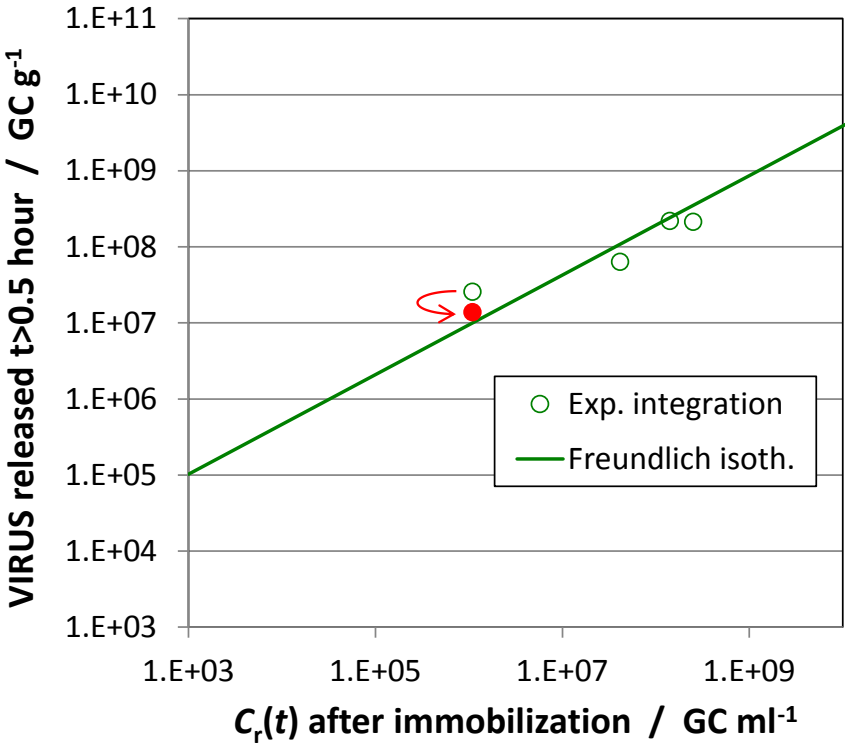

1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
[VIRUS] / GC ml-1
0
2
4
6
8
TIME / hour
Measured [virus] 0d
Fitted [virus] 0d
Measured [virus] 1d
Fitted [virus] 1d
Measured [virus] 2d
Fitted [virus] 2d
Measured [virus] 7d
Fitted [virus] 7d

Figure 3

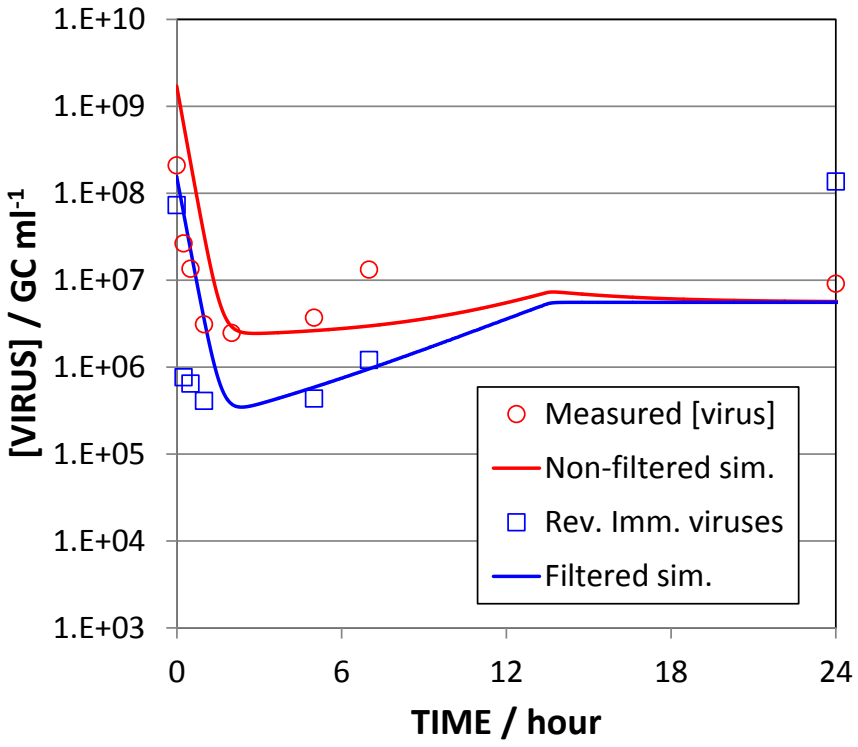

1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
VIRUS released t>0.5 hour / GC g-1
1.E+03
1.E+05
1.E+07
1.E+09
Cr(t) after immobilization / GC ml-1
Exp. integration
Freundlich isoth.

1 **Figure 4**

2

3

4



5

6

Modelling the removal and reversible immobilization of murine noroviruses in a phaeozem under various contamination and rinsing conditions

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Supporting information

Materials and Methods

Virus, soil and artificial soil solution

Table 1 Composition of circulating solutions

	Artificial soil solution	Groundwater	Sterilized wastewater
Organic C / mg l^{-1}	11.1	7.89	38.3
Ca^{2+} / mg l^{-1}	12.4	9.68	30.9
Mg^{2+} / mg l^{-1}	3.26	36.7	21.4
K^{+} / mg l^{-1}	13.8	64.7	25.4
Na^{+} / mg l^{-1}	10.9	58.6	12.5
NH_4^{+} / mg l^{-1}	0.35	0.96	0.40
NO_3^{-} / mg l^{-1}	2.07	0.15	0.53
Total N / mg l^{-1}	2.66	1.96	3.13
Cl^{-} / mg l^{-1}	5.23	53.3	92.0
Bore / mg l^{-1}	1.16	0.619	-
S (of SO_4^{2-}) / mg l^{-1}	6.61	23.2	21.8
pH	8.98	8.76	8.60

Materials and Methods

Experimental design and procedure

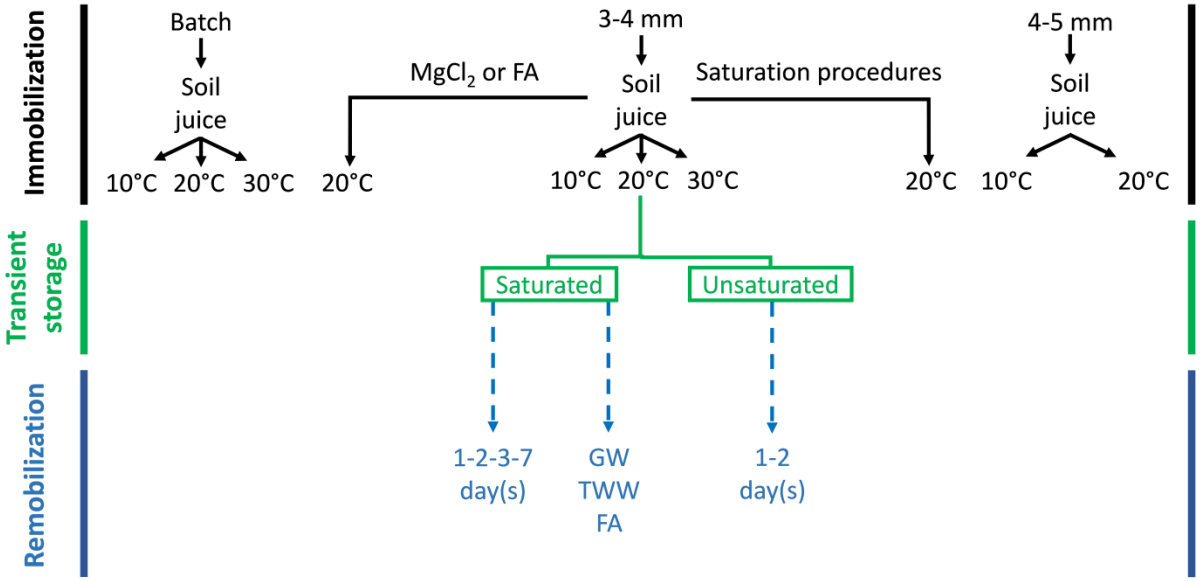
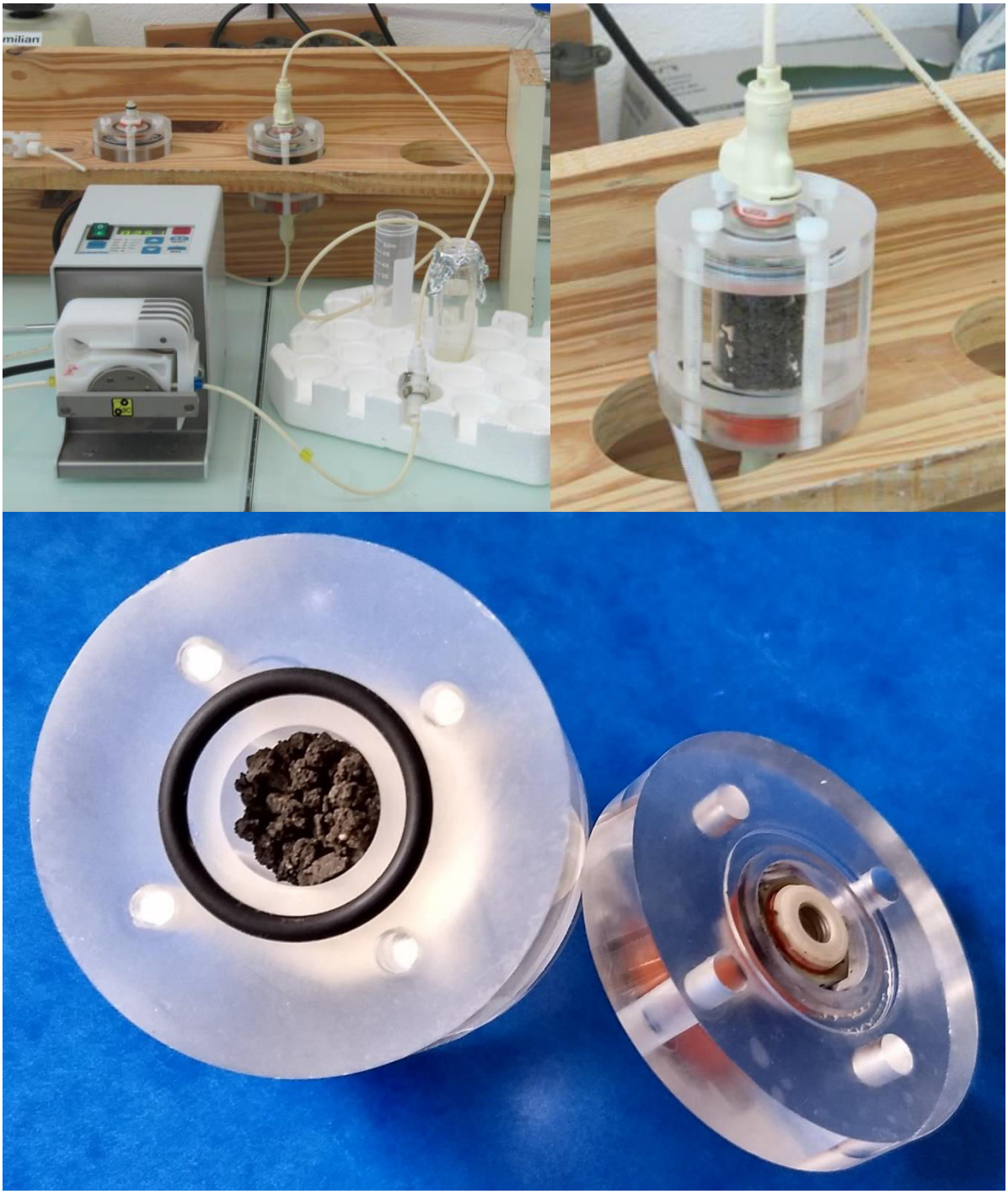


Figure S1 Experimental design of the study. GW: groundwater, TWW: treated wastewater, FA: fulvic acids.



Figures S2 Photos of (a) the experimental setup used for monitoring virus immobilization, (b) details of the column filled with soil aggregates, and (c) details of an open column.

Results and Discussion:

Reversible immobilization and irreversible removal in saturated conditions over a week

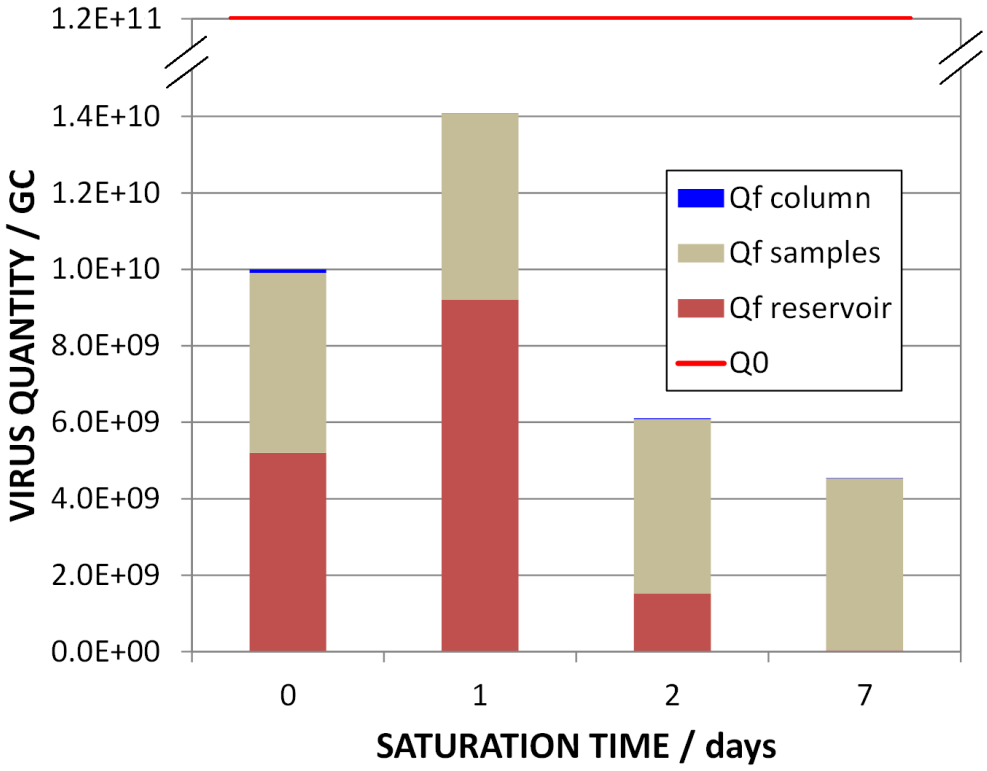


Figure S3 Quantities of viruses recovered from the sampled soil solution (Qf samples), the reservoir (Qf reservoir) and the soil aggregate columns (Qf column), compared with initial virus quantity contaminating the reservoir (Q0).

Results and Discussion:

Impact of soil drying on the reversibility of immobilizations

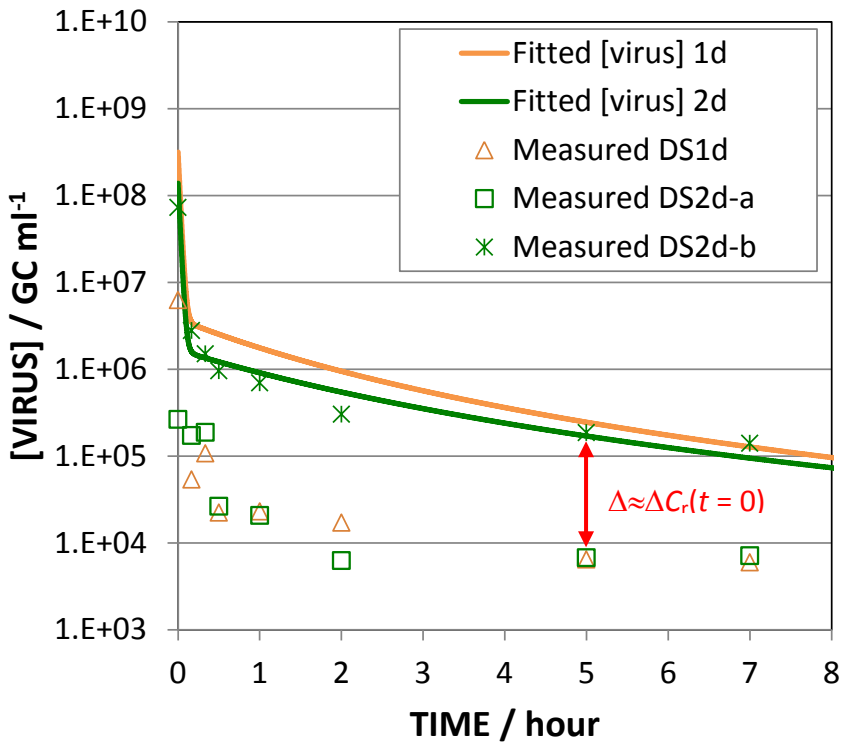


Figure S4 Experimental data for 7 hours virus remobilization experiments after a partial desaturation of the soil columns for 1 day (column DS1d with final $\omega = 0.36$ g g^{-1}) or 2 days (columns DS2d-a with final $\omega = 0.24$ g g^{-1} and column DS2d-b with final $\omega = 0.17$ g g^{-1}). (Simulations were performed using $C_r(t = 0)$, k_i, k_F and n_F estimated for immobilization and remobilization in columns always saturated (see values in caption of Figure 3a, b)).

Results and Discussion:

Impact of geochemistry on immobilization and subsequent desorption

Simulations of virus fate for artificial soil solution initially enriched with the equivalent of 0.01 mM $MgCl_2$ were carried out according to the following equations:

$$[\mathrm{Mg}^{2+}](t) = \left([\mathrm{Mg}^{2+}](t=0) - [\mathrm{Mg}^{2+}](t=\infty)\right) \times e^{-0.1\times t} + [\mathrm{Mg}^{2+}](t=\infty) \qquad \text{(S1)}$$

where t is the time since the beginning of the contamination (hour), $[Mg^{2+}](t = 0) = 10$ mM, and $[Mg^{2+}](t = \infty) = 3.1$ mM.

k_1, k_2 and k_D' were assumed to depend on $[Mg^{2+}]$:

$$k_1(t) = k_1(t=0) \times 10^{1-\frac{[\mathrm{Mg}^{2+}](t=0)}{[\mathrm{Mg}^{2+}](t)}} \qquad \text{(S2a)}$$

$$k_2(t) = k_1(t) \qquad \text{(S2b)}$$

$$k_D'(t) = k_D'(t=0) \times 10^{1-\frac{[\mathrm{Mg}^{2+}](t=0)}{[\mathrm{Mg}^{2+}](t)}} \qquad \text{(S2c)}$$

with $k_1(t = 0) = k_2(t = 0) = 10$, and $k_D' = 6\ 10^4$.

In addition k_c was set to $5\ 10^{-5}\ s^{-1}$.

Results and Discussion:

Impact of geochemistry on immobilization and subsequent desorption

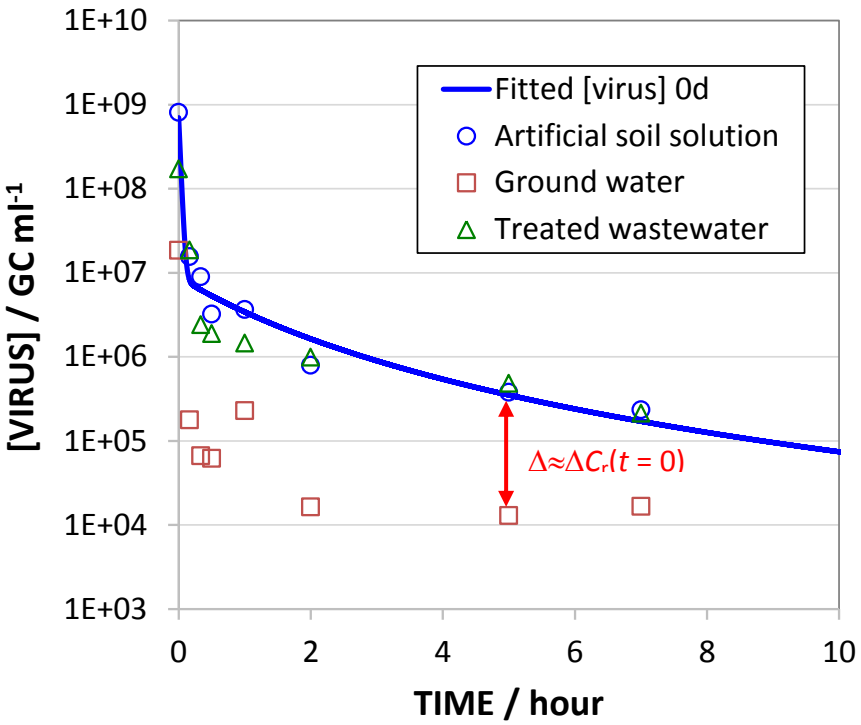


Figure S5 Experimental virus remobilization experiments for circulating artificial soil solution, autoclaved wastewater, and groundwater, after a first virus immobilization for soil aggregate columns at 20°C and exposed to contaminated artificial soil solution.

Annexe V

Reversible immobilization and irreversible removal of viruses in soils or mixtures of soil materials; an open data set enriched with a short review of main trends

* Cette Annexe correspond à une synthèse bibliographique déposée sur la base de données *HAL* du *CNRS*.

Reversible immobilization and irreversible removal of viruses in soils or mixtures of soil materials; an open data set enriched with a short review of main trends

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✉ Pierre Renault

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I. Introduction

Human enteric viruses brought to the soil by contaminated irrigation water can reach and contaminate plant or groundwater while remaining fully infective for weeks, and then cause waterborne and foodborne diseases. However, virus immobilization or inactivation within the soil may delay or prevent their transfer. In order to better assess those processes and the resulting sanitary hazards, models have been published. In this paper, we collected data from about 27 papers dealing simultaneously with reversible immobilization of viruses within the soil or in (mixtures of) soil material(s) (clay, sand ...) and irreversible virus removal, without always being able to distinguish between virus destruction and irreversible immobilization.

II. Reversible virus immobilisation

Some models distinguish kinetic reversible immobilization of free viruses in soil solution, from their kinetic remobilization:

$$\Phi_a = k_{a-r} \times \rho_b \times C_i \quad (1a)$$

$$\Phi_d = k_{d-r} \times \rho_b \times S_r \quad (1b)$$

where Φ_a and Φ_d are the adsorption and desorption fluxes of viruses per volume of bulk soil, respectively (mol day^{-1} ml^{-1}), ρ_b the bulk density of aggregates (g ml^{-1}), C_i the concentration of free viruses in soil solution (virus ml^{-1}), S_r the concentration of reversibly adsorbed viruses

(virus g^{-1}), k_{a-r} the coefficient for reversible adsorption on soil solids (ml g^{-1} day^{-1}), and k_{d-r} the coefficient for desorption of reversibly adsorbed viruses (day^{-1}).

For high k_{a-r} and k_{d-r} values and for a constant k_{a-r}-to-k_{d-r} ratio, C_i and S_r satisfy the following equilibrium:

$$\frac{S_r}{C_i} = \frac{k_{a-r}}{k_{d-r}} = k_D \tag{2}$$

where k_D is the partition coefficient (ml g^{-1}). The adsorbed virus concentration S_r is then proportional to the concentration of free viruses suspended in water C_i. Equation (2) may be expressed in decimal logarithms:

$$log(S_r) = log(k_D) + log(C_i) \tag{3}$$

Because this assumption is not valid when adsorption sites differ from each other and/or adsorption cannot exceed a maximum, other mathematical formalisms have been proposed.

Freundlich isotherm corresponds to the following relationship between C_i and S_r:

$$S_r = k_F \times C_i^{\frac{1}{n_F}} \tag{4}$$

where n_F is a parameter that indirectly describes the deviation from the proportional model involving k_D partition coefficient, and k_F a proportionality parameter. Equation (4) is often expressed in decimal logarithms:

$$log(S_r) = log(k_F) + \frac{1}{n_F} \times log(C_i) \tag{5}$$

As in the k_D partition model, there is no maximum adsorption limit in the Freundlich isotherm model.

Langmuir isotherm corresponds to the following relationship between C_i and S_r:

$$S_r = S_{max-r} \times \left(\frac{C_i}{\beta + C_i}\right) \tag{6}$$

where $S_{max\text{-}r}$ is the maximal concentration of reversibly adsorbed viruses (virus g^{-1}), and β the concentration of free viruses in soil solution (virus ml^{-1}) that leads to half of the reversible adsorption sites occupied. Equation (6) may be expressed in decimal logarithms:

$$log(S_r) = log(S_{max-r}) + log\left(\frac{C_i}{\beta + C_i}\right) \quad (7)$$

Main features of the models and the corresponding parameter values are reported in Tables 1 and 2. In Figure 1, adsorbed virus concentrations S_r are represented as a function of free virus concentrations C_i in soil solution. In Figure 2, the estimated S_r values for C_i=10^4 virus ml^{-1} are represented as a function of soil clay content.

44%, 28%, 36% and 4% of the publications mentioned in this paper employed the kinetic model, the k_D partition model, Freundlich isotherm and/or Langmuir isotherm, respectively; three of the publications compared two of these models (Table 1). For a fixed concentration of free viruses in soil solution (e.g. 10^4 virus ml^{-1}), deviation between minimum and maximum concentration of reversibly adsorbed viruses is about 6 $\log_{10}$ (see Figures 1 and 2). Most Freundlich isotherms use a n_F value very close to 1, making these isotherms *de facto* close to the k_D partition model. Due to the various forces involved in virus adsorption, it is difficult to highlight obvious relationships between variables.

Table 1: Main features of reversible immobilization models retained in published papers.

References	Reversible adsorption				Irreversible adsorption	
	Kinetic approach	K_D partition coefficient	Freundlich isotherm	Langmuir isotherm	From soil solution	From reversibly adsorbed pool
Burge & Enkiri (1978)			●			●
LaBelle & Gerba (1979)			●		●	
Vilker & Burge (1980)				●	●	
Moore et al. (1981)			●			●
Tim & Mostaghimi (1991) with experimental results from Lance & Gerba (1984)	●	●				●
Yates & Ouyang (1992) with experimental results from Grosser (1985); Ungs *et al.* (1985); Grondin (1987); Yates *et al.* (1988); Bales *et al.* (1989); Ouyang (1990)		●			●	
Grant et al. (1993)	●			●		●
Powelson & Gerba (1994)		●	●		●	
Dowd et al. (1998)	●	●			●	
Thompson et al. (1998)			●			●
Sim & Chrysikopoulos (1999) with experimental results from Hurst et al. (1980)		●			●	
Gantzer et al. (2001)			●		●	
Schijven & Šimůnek (2002) with experimental results from Schijven *et al.* (1999, 2000)	●				●	
Flynn et al. (2004)	●				●	
Torkzaban et al. (2006)		●			●	
Cheng et al. (2007)		●			●	
Zhao et al. (2008)			●		●	
Zhuang & Jin (2008)		●			●	
Anders & Chrysikopoulos (2009)		●			●	
Cao et al. (2010)	●					●
Syngouna & Chrysikopoulos 2010)		●			●	
Chrysikopoulos & Syngouna (2012)			●		●	
Sadeghi et al. (2013)	●					●
Chrysikopoulos & Aravantinou (2014)			●		●	
Mayotte et al. (2017)	●					●
Sasidharan et al. (2017)	●				●	
Our upcoming study			●			●

Table 2: Parameter values used in published reversible immobilization models.

References	Soil type	Virus species	Conc. range or punctual value ($\log_{10}$ virus ml^{-1})[a]	K_d ($ml\ g^{-1}$)	Kinetic approach		Freundlich isotherm		Langmuir isotherm	
					k_{att} (h^{-1})	k_{det} (h^{-1})	K_F	n_F	S_r	β
Chrysikopoulos & Aravantinou (2014)	Quartz sand	Φ174	3-8				1.55	1.15		
							2.57	1.10		
							1.15	1.05		
							0.14	0.97		
							0.52	1.09		
							0.79	1.18		
							0.3	1.08		
							0.52	1.16		
							0.63	1.06		
		MS2	3-8				2.06	1.18		
							3.54	1.41		
							3.55	1.16		
							1.54	1.04		
							2.22	1.18		
							8.51	1.18		
							0.57	1.16		
							2.12	1.33		
							3.08	1.27		
Chrysikopoulos & Syngouna (2012)	Kaolinite	Φ174	3-9				2260	1.00		
		MS2	3-9				758	0.89		
	Montmorillonite	Φ174	3-9				271	1.08		
		MS2	3-9				4340	0.94		
Thompson *et al.* (1998)	Loamy sand	Φ174	2-7				2.89	1.09		
							1.01	1.16		
		MS2	2-7				0.076	0.99		
	Sandy loam	Φ174	2-7				6.52	1.02		
							10.99	1.06		
		MS2	2-7				0.437	1.01		
	Sand	Φ174	2-7				0.44	0.99		
LaBelle & Gerba (1979)	Sediments	Echo 1	4-5				$10^{4.77}$	1.24		
		PV1	4-5				$10^{5.42}$	3.45		

References	Soil type	Virus species	Conc. range or punctual value ($\log_{10}$ virus ml^{-1})[a]	K_d ($ml\ g^{-1}$)	Kinetic approach		Freundlich isotherm		Langmuir isotherm	
					k_{att} (h^{-1})	k_{det} (h^{-1})	K_F	n_F	S_r	β
Cao *et al.* (2010)	Sandy soil	MS2	2.5		0.06	6000				
					0.06	$10^{12.78}$				
					1.20	12.00				
					16.14	46.38				
					22.56	56.64				
					16.62	54.24				
					15.24	28.80				
					0.06	6000				
					0.06	$10^{12.78}$				
					3.48	43.62				
					4.62	13.26				
					5.76	32.76				
					6.18	52.32				
					7.32	24.06				
Zhao *et al.* (2008)	Loam	MS2	2-7				0.228	0.96		
							1.016	0.97		
Sasidharan *et al.* (2017)	Quartz sand	Φ174	6.7		0.02	0.04				
					0.07	0.02				
					0.07	0.06				
					0.07	0.02				
		PRD1	6.7		0.16	0.06				
					0.02	0.03				
					0.38	0.10				
					0.33	0.09				
Dowd *et al.* (1998)	Aquifer sand	MS2	9.3		19.08	3.96				
		PRD1	10.5		19.44	4.32				
		Qβ	4.8		24.48	1.44				
		ϕ 174	6.3		10.44	5.40				
		PM2	7.2		8.64	0.72				

References	Soil type	Virus species	Conc. range or punctual value ($\log_{10}$ virus ml^{-1})[a]	K_d ($ml\ g^{-1}$)	Kinetic approach		Freundlich isotherm		Langmuir isotherm	
					k_{att} (h^{-1})	k_{det} (h^{-1})	K_F	n_F	$S_{r\,max}$	β
Burge & Enkiri (1978)	Clay loam	Φ174	2-7				72.5	1.06		
	Silt loam	Φ174	2-7				4.61	1.09		
							161	1.10		
							45.7	0.81		
Moore *et al.* (1981)	Ottawa sand	PV	8-9				0.631	0.83		
Vilker & Burge (1980)	Kranzburg soil	Φ 174	4.2-7.2						$10^{8.32}$	$10^{10.30}$
			7.1-10.5						$10^{8.28}$	$10^{10.98}$
Powelson & Gerba (1994)	Sandy alluvium	PV	3-6	160.0						
		MS2	3-6	3.70						
		PRD1	3-6	16.00						
Schijven & Šimůnek (2002) with experimental results from Schijven *et al.* (1999, 2000)	Dune sand	MS2	7.9		0.17	$10^{-4.44}$				
					0.13	$10^{-4.18}$				
					0.12	$10^{-3.97}$				
					0.08	$10^{-4.12}$				
					0.05	$10^{-4.66}$				
					0.03	$10^{-3.90}$				
		PRD1	7		0.17	$10^{-4.49}$				
					0.13	$10^{-4.34}$				
					0.09	$10^{-4.12}$				
					0.06	$10^{-3.98}$				
					0.05	$10^{-4.06}$				
					0.03	$10^{-3.85}$				
Sim & Chrysikopoulos (1999) with experimental results from Hurst *et al.* (1980)	Loamy sand	PV1	4	0.087						

References	Soil type	Virus species	Conc. range or punctual value (log_{10} virus ml^{-1})[a]	K_d (ml g^{-1})	Kinetic approach		Freundlich isotherm		Langmuir isotherm	
					k_{att} (h^{-1})	k_{det} (h^{-1})	K_F	n_F	S_r	β
Syngouna & Chrysikopoulos 2010)	Kaolinite	Φ174	3-9	16.00						
				36.00						
				21.00						
				24.00						
	Montmorillonite	MS2	3-9	78.00						
				68.00						
				19.00						
				21.00						
Tim & Mostaghimi (1991) with experimental results from Lance & Gerba (1984)	Loamy sand	PV1	4	1200						
Cheng *et al.* (2007)	Sand	MS2	6-9		0.91	0.06				
					0.29	0.01				
					0.35	0.003				
					0.64	0.01				
					0.53	0.01				
Mayotte *et al.* (2017)	River sand	MS2	8.04-8.34		0.46	0.14				
					0.24	0.21				
					2.08	$10^{-5.08}$				
					0.72	0.13				

References	Soil type	Virus species	Conc. range or punctual value (log_{10} virus ml^{-1})[a]	K_d ($ml\ g^{-1}$)	Kinetic approach		Freundlich isotherm		Langmuir isotherm	
					k_{att} (h^{-1})	k_{det} (h^{-1})	K_F	n_F	S_r	β
Flynn *et al.* (2004)	Fresh kappelen sands	H 40/1	2.6		$10^{4.06}$	-				
	Quartz sands	H 40/1	2.6		7290	-				
	Granitic sands	H 40/1	2.6		1800	-				
	Quartz-calcite mixture	H 40/1	2.6		3480	-				
	Reused kappelen sands	H 40/1	2.6		1080	-				
	Washed kappelen sands	H 40/1	2.6		$10^{4.41}$	-				
	Acid-digested sands	H 40/1	2.6		960	-				
Sadeghi *et al.* (2013)	Quartz sand	PRD1	4.5-5.2		0.19	0.026				
					0.086	0				
					0.21	0.014				
					1.20	$10^{-2.85}$				
					0.59	$10^{-4.57}$				
					1.10	$10^{-3.43}$				
					0.74	$10^{-3.04}$				
					2.10	$10^{-2.44}$				
Gantzer *et al.* (2001)	Cultivated soil	Coli-phages	1.3-2.9				4.34	0.80		
		F-RNA phages	0.6-2.6				40.74	1.11		

References	Soil type	Virus species	Conc. range or punctual value (log_{10} virus ml^{-1})[a]	K_d (ml g^{-1})	Kinetic approach		Freundlich isotherm		Langmuir isotherm	
					k_{att} (h^{-1})	k_{det} (h^{-1})	K_F	n_F	S_r	β
Torkzaban *et al.* (2006)	Quartz sand	MS2	6		6.00	2820				
					7.80	1800				
					6.00	1200				
					30.00	1320				
					48.00	1500				
					222	1.80				
					306	0.60				
					384	0.60				
		Φ 174	6		25.80	1500				
					49.20	420.00				
					492	84.00				
					780	90.00				
					900	102				
Anders & Chrysikopoulos (2009)	Monterey sand	MS2	5.74	33.72						
				380.9						
				136.7						
		PRD1	5.74	36.17						
				311.9						
				40.75						
Zhuang et al 2008	Goethite-sand mixture	ϕ 174	8.7	0.319						
				0.138						
				0.093						
				0.197						
		MS2	8.7	0.045						
				0.306						
				0.013						
				0.588						
Our upcoming study	Calcaric phaeozem	MNV	3-6				1120	1.53		

[a] Virus concentration in our study are expressed in genomic copies per ml (GC ml^{-1})

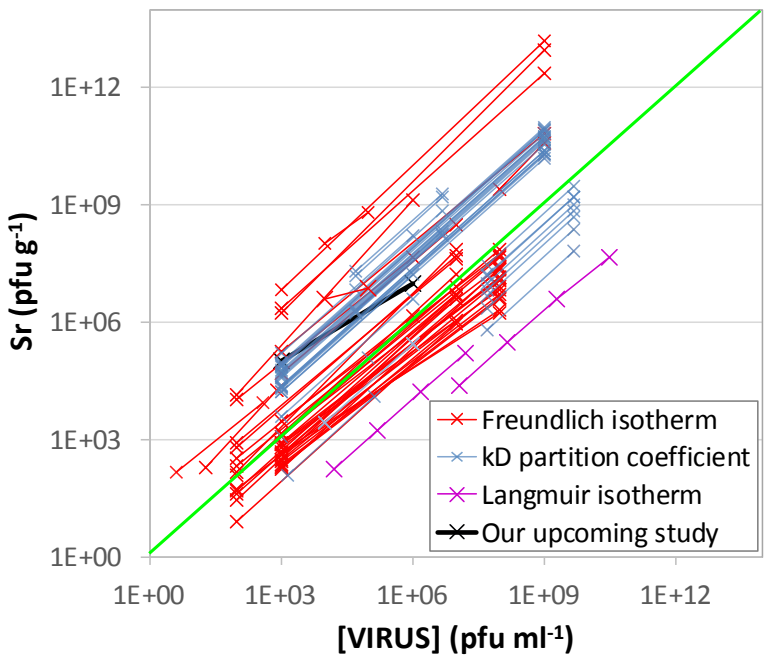

Figure 1: Published relationships between adsorbed virus concentrations S_r and free virus concentrations C_i in soil solution. Curves were plotted either in the given range of virus concentrations C_i, or when a unique C_i was experimented (especially for k_D partition models), between one tenth and ten times this value.

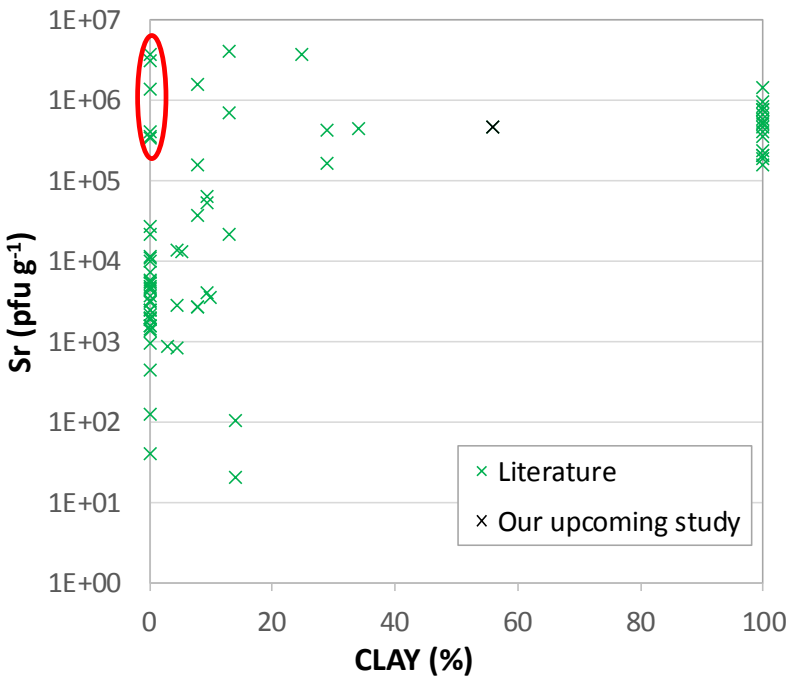

Figure 2: Relationships between reversibly adsorbed virus concentrations S_r for free virus concentrations $C_i = 10^4$ virus ml^{-1}, and soil clay contents (%) that were reported or estimated from the soil textural triangle. (Red circle accounts for iron oxide supplemented sands).

III. Irreversible virus removal

Among the 27 papers dealing with reversible virus adsorption, irreversible virus removal could be neglected over periods not exceeding the duration of reversible virus adsorption experiments in 4 papers (Dowd *et al.*, 1998; Cao *et al.*, 2010; Syngouna & Chrysikopoulos, 2010; Sasidharan *et al.*, 2017). In addition, results were not clear enough in five other papers (Burge & Enkiri, 1978; Chrysikopoulos & Syngouna, 2012; Chrysikopoulos & Aravantinou, 2014), and the estimated inactivation coefficients were probably biased due inappropriate partition coefficient k_D for reversible adsorption in another paper (Yates & Ouyang, 1992). Lastly, inactivation resulted mainly from the triple phase (air-liquid-solid (tube wall)) boundary in a last paper (Thompson *et al.*, 1998). Therefore, the following analysis focuses on the other 17 publications.

When irreversible virus removal was taken into account, it was described by first order kinetics. For irreversible removal from the soil solution, this may be written:

$$\frac{\partial C_i}{\partial t} = -\lambda \times C_i \tag{8}$$

where λ is a kinetic coefficient (day^{-1}). This equation leads to:

$$C_i(t) = C_i(t=0) \times e^{-\lambda t} = C_i(t=0) \times 10^{-\frac{\lambda}{Ln(10)}t} \tag{9}$$

For irreversible removal from the viruses reversibly adsorbed on the soil, equation (8) could be transposed as:

$$\frac{\partial S_r}{\partial t} = -\lambda \times S_r \tag{10}$$

When the quantity of reversibly adsorbed viruses can be described by the k_D partition model, the irreversible removal of reversibly adsorbed viruses leads to simultaneous change of virus concentration in soil solution according to equation (8) with the same rate coefficient λ.

When the quantity of reversibly adsorbed viruses can be described by Freundlich isotherms, the irreversible removal of reversibly adsorbed viruses leads to the simultaneous change of virus concentration in soil solution according to the following equation:

$$\frac{\partial C_i}{\partial t} = -\lambda \times n_F \times C_i \tag{11}$$

The coefficient rates of the published first order rates models of virus irreversible removals are reported in Table 3, as well as the durations of the corresponding experiments and/or simulations. In addition, coefficient rate values are reported as a function of the iron oxides of the corresponding soil or soil material(s) in Figure 3.

85 non-zero inactivation rate coefficients were reported in Table 3. They varied between 0.001 and 864 day^{-1}. No significant relationships were noted between coefficient rate values and other values that could affect it, e.g. with the soil clay content (Figure 3), probably because it depends on the simultaneous validation of different condition.

Table 3: Daily removal coefficient rates (λ) reported in literature.

References	Soil type	Virus species	λ (day^{-1})	Experiment duration (h)
Zhao et al. (2008)	Ferriudic cambosols	MS2	0.032	1320
		MS2	0.015	1320
		MS2	0.015	1320
		MS2	0.011	1320
	Ustandic primosols	MS2	0.008	1320
		MS2	0.006	1320
		MS2	0.01	1320
		MS2	0.007	1320
Gantzer et al. (2001)	Cultivated soil	Coliphages	0.131	240
		Coliphages	0.017	240
		Coliphages	0.049	240
		F-RNA	0.067	240
		F-RNA	0.075	240
		F-RNA	0.136	240
LaBelle & Gerba (1979)	Sediment	PV1	864.0	0.5
		Cox B4	864.0	0.5
		Echo 1	864.0	0.5
		Echo 7	864.0	0.5
		Rotavirus	864.0	0.5
Powelson & Gerba (1994)	Sandy alluvium	MS2	0.079	72
		PRD1	0.167	72
		PV1	0.719	72
Sim & Chrysikopoulos (1999) with experimental results from Hurst et al. (1980)	Loamy sand	PV1	0.0	1800
Tim & Mostaghimi (1991) with experimental results from Lance & Gerba (1984)	Loamy sand	PV1	2.22	96
Anders & Chrysikopoulos (2009)	Monterey sand	MS2	0.026	2
		MS2	0.066	2
		PRD1	0.001	2
		PRD1	0.002	2
Schijven & Šimůnek (2002) with experimental results from Schijven *et al.* (1999, 2000)	Dune sand	MS2	0.017	960
		MS2	0.071	960
		MS2	0.024	960
		MS2	0.049	960
		MS2	0.053	960
	Dune sand (Well 1-6)	MS2	0.013	2880
	Dune sand (Well 1)	MS2	0.037	2880
	Dune sand (Well 2-6)	MS2	0.040	2880
	Dune sand (Well 1-6)	PRD1	0.052	2880
	Dune sand (Well 1)	PRD1	0.031	2880
	Dune sand (Well 2-6)	PRD1	0.029	2880
Cheng et al. (2007)	Vinton soil	MS2	2.88	50
		MS2	5.76	50
		MS2	6.34	50
Mayotte et al. (2017)	River sand (new)	MS2	0.190	1440
	River sand (new)	MS2	0.083	1440
	River sand (used)	MS2	0.065	1440
	River sand (used)	MS2	0.083	1440
	River sand (new)	MS2	0.013	1440
	River sand (used)	MS2	112.6	1440
Vilker & Burge (1980)	Kranzburg soil	Φ 174	0.620	48

References	Soil type	Virus species	λ (day^{-1})	Experiment length (h)
Dowd *et al.* (1998)	Aquifer sand	MS2	0.02	4
		PRD1	0.03	
		Qβ	0.08	
		ϕ 174	0.42	
		PM2	0.58	
Flynn et al. (2004)	Fresh kappelen sands	H40/1	118.82	3
	Quartz sands	H40/1	11.26	3
	Granitic sands	H40/1	10.01	3
	Quartz-calcite mixture	H40/1	265.16	3
	Reused kappelen sands	H40/1	75.98	3
	Washed kappelen sands	H40/1	18.76	3
	Acid-digested kappelen sands	H40/1	36.27	3
Sadeghi et al. (2013)	Quartz sand	PRD1	0.07	30
		PRD1	0.07	30
		PRD1	0.53	30
Torkzaban et al. (2006)	Sand	MS2	0.02	6
		ϕ 174	0.01	6
Powelson et al. (1993)	Sandy alluvium	MS2	2.40	6
		MS2	2.71	6
		MS2	3.86	6
		MS2	1.35	6
		MS2	2.40	6
		PRD1	6.77	6
		PRD1	10.42	6
		PRD1	3.65	6
		PRD1	5.94	6
		PRD1	6.15	6
		PRD1	8.55	6
Zhuang & Jin (2008)	Goethite-coated sand	ϕ 174	4.59	1320
		ϕ 174	13.55	1320
		ϕ 174	1.25	1320
		ϕ 174	5.73	1320
		MS2	0.21	1320
		MS2	8.13	1320
		MS2	3.13	1320
		MS2	4.69	1320
Our upcoming study	Calcaric phaeozem	MNV	0.38	7

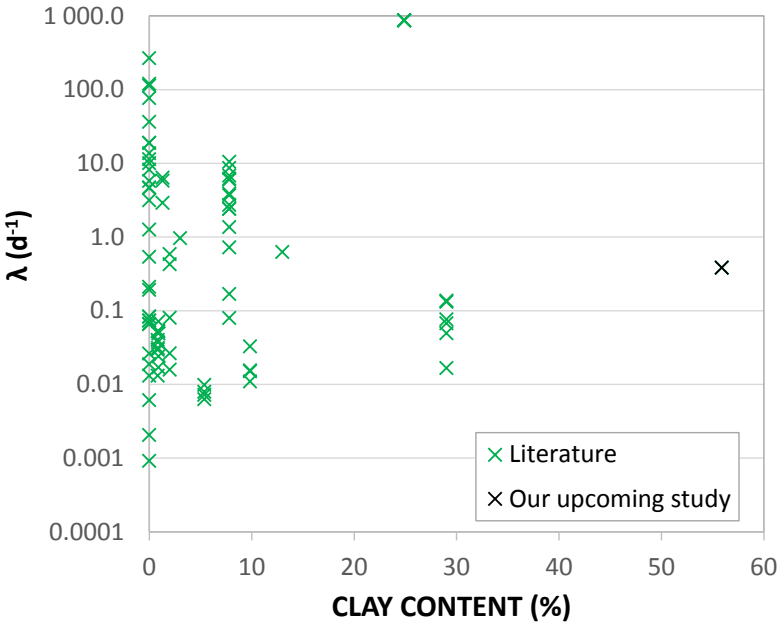

Figure 3: Daily removal rates as reported in literature

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Annexe VI

Résumé vulgarisé de la thèse

* Cette Annexe correspond à un résumé vulgarisé de la thèse à des fins de diffusion auprès du monde professionnel et en particulier des acteurs professionnels impliqués dans ce travail.

Eaux usées ; une vie après la vie ? Les risques viraux associés à divers modes de gestion

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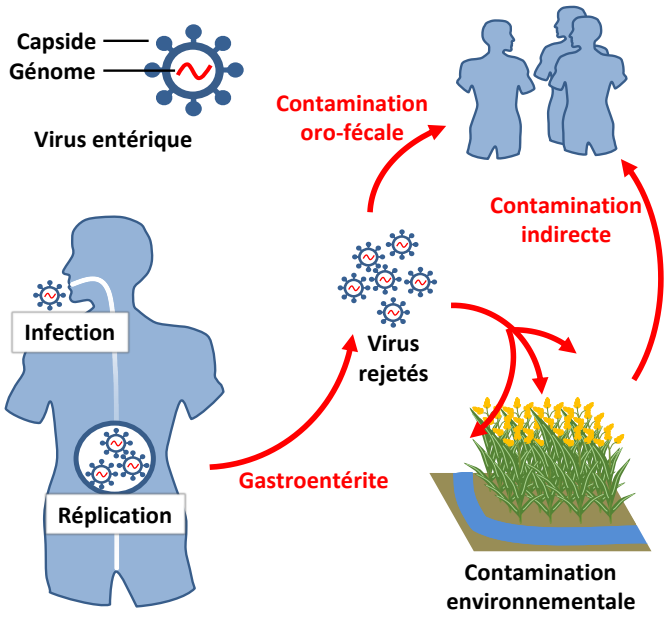
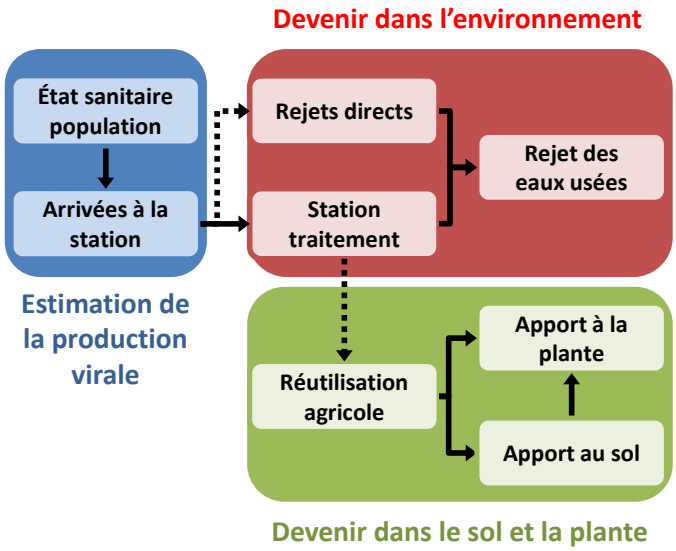
Avec la diminution des ressources en eaux douces et leur contamination par les rejets d'eaux usées non ou insuffisamment traitées émerge la nécessité d'une gestion plus durable de ces ressources dans un contexte mondial d'accroissement et d'urbanisation des populations, de diversification des usages de l'eau et de réchauffement climatique. La réutilisation des eaux usées peut aider à relever ce défi en diminuant leur impact sur la pollution des eaux douces et en accroissant localement les quantités d'eau mobilisable. Lorsqu'elle soutient l'irrigation agricole, elle contribue aux fertilisations phosphatée et azotée et autorise en climat chaud la succession annuelle de plusieurs cultures sur une même parcelle. Mais la filière agricole peut être en compétition avec des filières industrielles ou urbaine de réutilisation d'eau usée, voire avec la fourniture d'eau potable. La réutilisation des eaux usées est importante aux Proche et Moyen Orients, dans les pays méditerranéens, en Australie, dans le sud-ouest des Etats-Unis, en Chine et au Japon. En Europe, elle concerne d'abord Chypre, Malte, l'Espagne et l'Italie et, dans une moindre mesure, la Grèce avec la Crète et la Turquie. En France, elle sert à irriguer moins de 2500 ha (cultures, terrains de golf, parcs) dans le Sud, en régions côtières, dans des îles, et dans des terres intérieures ayant un accès difficile aux ressources en eau. La présence dans les eaux usées de polluants chimiques et biologiques divers génère des risques sanitaires, agricoles et environnementaux qu'il convient d'évaluer.



Structure d'un virus entérique et cycle de contamination

Les virus entériques pathogènes de l'Homme sont des contaminants microbiologiques que l'*U.S. - Environmental Protection Agency* et l'*American Water Works Association* suggèrent de suivre dans les eaux potables et que la Commission du *Codex Alimentarius* reconnait comme l'un des risques microbiens les plus importants. Il s'agit de virus de quelques dizaines de nanomètres, dits "nus" c.à.d. combinant un génome viral constitué d'ADN ou d'ARN protégé par une capside de protéines. *(Les virus enveloppés (par ex. virus de la grippe et virus de l'herpès) possèdent autour de leur capside une membrane issue de la cellule hôte)*. Les virus entériques de l'Homme incluent le virus de l'hépatite A, les norovirus, rotavirus, entérovirus, adénovirus …. Ils sont à l'origine de gastroentérites aiguës, d'hépatites et de dommages au foie, de fièvres, et parfois de méningites, de troubles du système nerveux, de maladies respiratoires, de maladies cardio-vasculaires, d'infections des yeux et de maladies congénitales. Si la gastroentérite aiguë est le symptôme le plus fréquent des infections par la plupart d'entre eux, son impact peut être fatal aux populations fragiles qui représentent 20 à 25% de la population des pays industrialisés (enfants de moins de 5 ans, adultes de plus de 65 ans, personnes immunodéprimées, diabétiques, femmes enceintes). Les virus entériques pathogènes de l'Homme ne se répliquent qu'après avoir pénétré une cellule vivante en utilisant la machinerie cellulaire de cette dernière. Ils peuvent être excrétés en très grande quantité (jusqu'à 100 milliards de virus par gramme de selle) et sur des périodes dépassant la durée des symptômes (jusqu'à 80 jours) par les malades et les porteurs sans symptômes. La transmission oro-fécale prédomine mais la contamination par l'eau, les aliments ou l'air est possible ; elle est favorisée par la grande résistance des virus dans l'environnement et aux traitements de potabilisation de l'eau. En rivières, les concentrations en norovirus peuvent atteindre 10 000 norovirus par litre. Longtemps supposées saines, les eaux souterraines et les eaux de sources peuvent aussi être contaminées.



Devenir des virus de la personne infectée aux milieux récepteurs des eaux usées

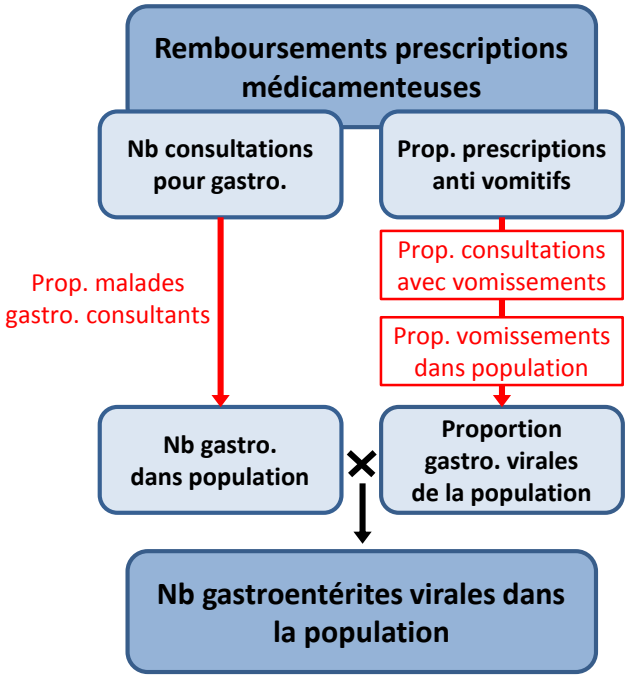
Quelques particules virales (10 à 100) suffisent pour infecter une personne, les virus excrétés n'étant pas tous infectieux et certains pouvant perdre leur pouvoir infectieux dans l'environnement.

La comparaison des risques viraux associés à divers modes de gestion des eaux usées peut contribuer à évaluer l'acceptabilité sanitaire de ces derniers. On peut envisager des scénarios se distinguant par la collecte des eaux usées et leurs traitements, le stockage éventuel des eaux traitées en réservoir, leur rejet dans l'environnement ou leur réutilisation. Pour une réutilisation en irrigation agricole, les risques varient avec le type de culture, les caractéristiques des irrigations, le délai entre la dernière irrigation contaminante et la récolte, l'histoire de la culture sur cette période et après la récolte. L'évaluation quantitative des risques microbiens est l'évaluation de la probabilité d'occurrence d'une maladie, voire l'évaluation probabiliste de ses effets néfastes sur la santé dans un contexte donné. Ces effets, exprimés en *"années de vie en bonne santé perdues"*, cumulent les pertes dues à une mortalité précoce et la durée des cas de maladies non mortelles pondérée par leur caractère plus ou moins invalidant. A titre indicatif, une gastroentérite à norovirus équivaut à 0,012 années de vie en bonne santé perdue ; un individu sur 3 étant atteint de gastroentérite aiguë chaque année, cela amène à environ 0,004 années de vie en bonne santé perdue par personne et par an due aux gastroentérites aiguës. Eu égard aux impacts sanitaires de la seule réutilisation des eaux usées en irrigation agricole, l'*Organisation Mondiale de la Santé* préconise un seuil toléré beaucoup plus faible (de 0,000001 à 0,0001 année de vie en bonne santé perdue par personne et par an) correspondant à une contribution négligeable de cette pratique à l'ensemble des cas de gastroentérites. La probabilité de survenue d'une maladie combine la probabilité de contamination d'un individu (le pathogène atteint les organes cibles), la probabilité d'infection qui suit (le pathogène est actif), et la probabilité que des symptômes en découlent. Leur évaluation peut avoir une composante aléatoire pour tenir compte de la variabilité des expositions et des réponses des individus au pathogène. L'évaluation de l'exposition des personnes à des virus infectieux suppose de pouvoir quantifier en amont la production de virus entériques pathogènes de l'Homme et leur devenir ultérieur (transfert, immobilisation ou rétention sur des phases solides, inactivation (c.à.d. la perte de leur pouvoir infectieux)) dans tous les compartiments où le virus peut essaimer : égout, station d'épuration des eaux usées, environnement (eau, sol, production végétale, air …). Il y a un manque de connaissances sur ces derniers aspects justifiant les travaux résumés dans la suite de cet article.

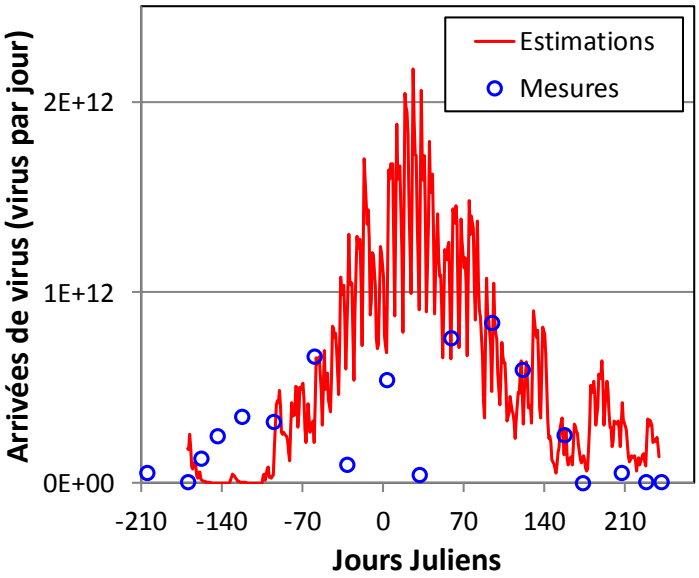
Estimer l'excrétion virale journalière sur

des périodes annuelles est intéressant pour permettre une évaluation quantitative des risques après avoir simulé le devenir ultérieur des virus dans les égouts, en station d'épuration et dans les milieux récepteurs des eaux traitées. Accessibles en temps réel, ces données permettraient aussi d'adapter les traitements en station. Malheureusement, le suivi journalier des virus entériques pathogènes de l'Homme dans les eaux usées est empêché par les coûts et la durée des analyses, et les indicateurs règlementaires suivis pour la réutilisation ou le rejet d'eaux usées ne reflètent pas les concentrations en virus entériques pathogènes de l'Homme.

Une alternative possible est d'avoir accès au nombre de gastroentérites aiguës provoquées par des virus et d'associer à chacune une excrétion virale journalière qui dépend du temps écoulé depuis le début de l'infection. Sachant que la fréquence de certains symptômes (vomissement, diarrhée muqueuse, fièvre) varie avec l'étiologie des gastroentérites aiguës (c.à.d. avec l'agent



Mode d'évaluation des nouveaux cas journaliers de gastroentérites aiguës à partir des prescriptions de médicaments remboursés par l'*Assurance Maladie*.



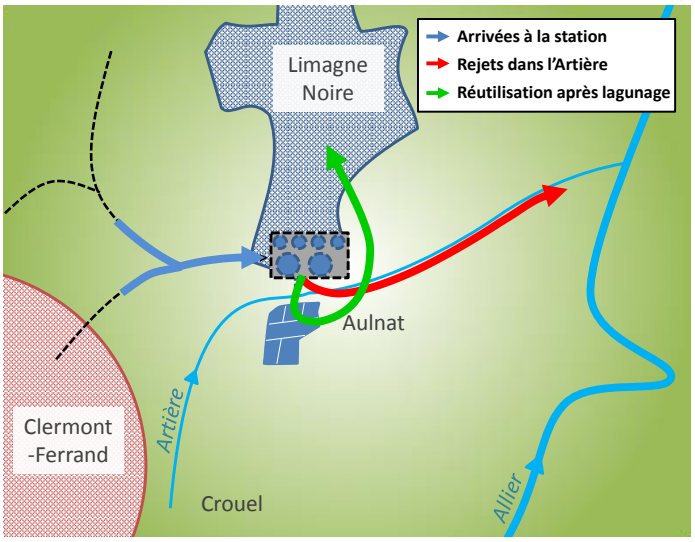
Entrée de station d'épuration *'Les Trois* Rivières' : prélèvement d'1 L eau usée, filtration ultérieure des virus au laboratoire, et confrontation entre quantités journalières de virus simulées et évaluées à partir des mesures *in situ*.

infectieux qui en est la cause), nous avons utilisé les remboursements de prescriptions médicamenteuses mises à notre disposition par l'agence *'Santé Publique France'* et l'*Assurance Maladie* pour la région clermontoise. Nous avons ainsi évalué la fréquence de vomissements dans la population consultant un médecin, puis dans la population globale souffrant de gastroentérite aiguë en tenant compte du fait que ce symptôme pousse à la consultation. Cette dernière fréquence nous a permis de calculer, pour chaque jour, une proportion puis un nombre de nouveaux cas de gastroentérites aiguës d'étiologie virale. Nous avons alors simulé les excrétions virales journalières associées ainsi que celles des personnes infectées ne présentant pas de symptôme, sans distinguer d'espèces virales, en utilisant un modèle d'excrétion. Les concentrations en norovirus génogroupes I et II, rotavirus, entérovirus et adénovirus ont été suivies expérimentalement en entrée de station d'épuration, avec une fréquence mensuelle ou bimensuelle. Après les avoir pondérées pour refléter les contributions spécifiques de chaque espèce virale aux gastroentérites aiguës, la somme de ces concentrations a été comparée aux valeurs simulées.

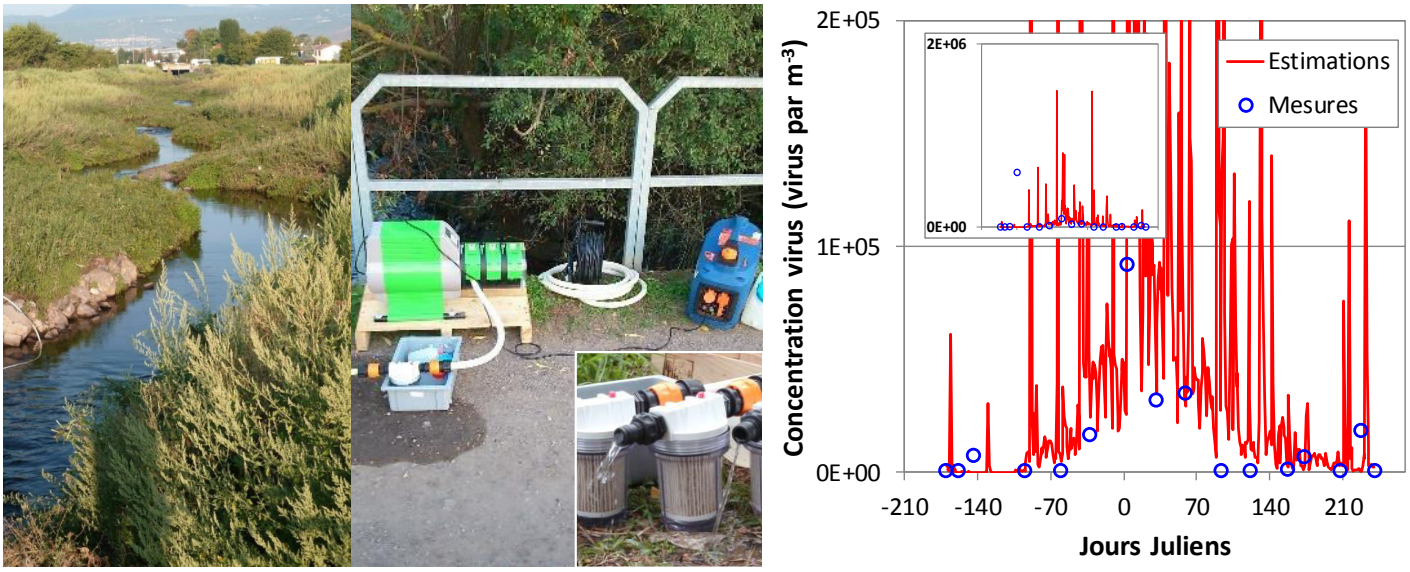
La comparaison des quantités simulées de virus arrivant à la station d'épuration à la somme pondérée des quantités détectées y arrivant aussi confirme que l'on peut ainsi simuler des valeurs et des tendances cohérentes avec les valeurs expérimentales, avec un maximum hivernal et un minimum estival, généralement non nul.

Le degré d'accord entre simulations et valeurs expérimentales reste pourrait être affiné sur plusieurs aspects : prise en compte du devenir des virus dans les égouts, utilisation simultanée d'autres indicateurs discriminant les étiologies virales et bactériennes, étude sur des populations de plus grande taille.

En rivière, les quantités de virus amenés par les rejets d'eaux usées traitées peuvent alors être évaluées en tenant compte de l'abattement viral en station d'épuration, des quantités d'eaux usées traitées rejetées après le recyclage d'une partie de ces eaux pour une réutilisation en irrigation agricole, des délestages des eaux d'égouts unitaires en amont de la station d'épuration et/ou de l'activation du by-pass de la station d'épuration lors d'épisodes de précipitations importants. Le passage de ces quantités à des concentrations tient alors compte du débit de la rivière et de sa contamination en amont du point de rejet, ainsi que des volumes d'eau associés au rejet et éventuellement au by-pass, tant que les disparitions de virus peuvent être négligées. A noter que la concentration virale dans l'Artière en amont du point de rejet de la station d'épuration tient intègre les rejets d'eau usée traitée par une très petite station d'épuration en amont et, parfois de délestages du réseau d'égouts de Clermont-Ferrand.



Devenir des eaux usées associées à la station *'Les Trois Rivières'*. S'y ajoutent parfois des délestages d'égouts et le by-pass de la station.



Artière en aval des rejets d'eaux usées traitées de la station d'épuration *'Les Trois Rivières'* : Prélèvement et filtration *in situ* de 100 L d'eau ; confrontation entre concentrations journalières de virus simulées et évaluées à partir des mesures *in situ*.

Les concentrations en norovirus génogroupes I et II, rotavirus, entérovirus et adénovirus ont été suivies expérimentalement en sortie de station d'épuration sur des échantillons d'1 L d'eau, tandis qu'elles ont été suivies sur des volumes de 100 L d'eau pour les eaux de rivière (Artière et Allier) et pour les eaux des nappes alluviales en bordures d'Artière (au niveau de jardins potagers) et d'Allier (dans des puits servant à l'irrigation de maïs par pivots).

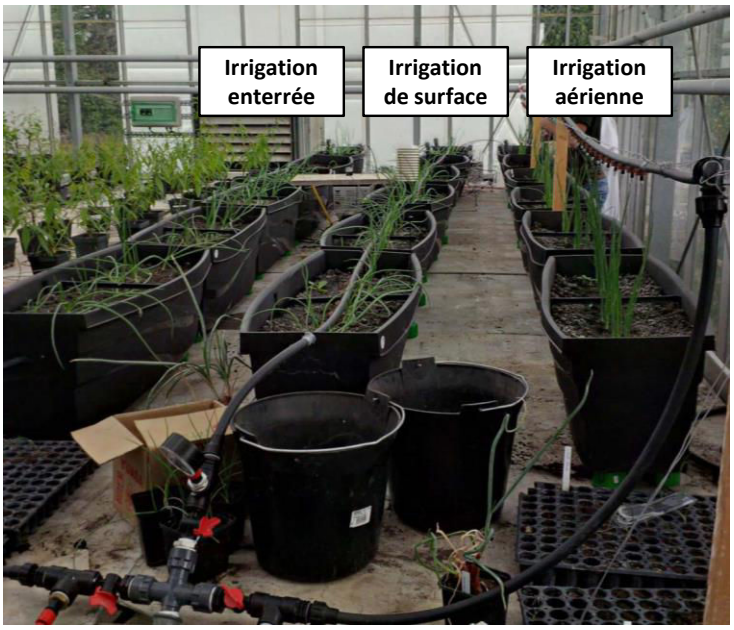
La confrontation des concentrations simulées aux concentrations relevées sur le terrain montre la cohérence entre les deux types de valeurs. Les simulations démontrent par ailleurs bien l'impact des by-pass sur la pollution virale de l'Artière. A noter qu'une campagne ponctuelle de mesures dans jardins potagers en bordure d'Artière a montré que les concentrations virales dans les cultures potagères et dans l'eau de nappe servant à irriguer ces jardins étaient toutes en-deçà des seuils de détection. Les suivis pratiqués sur l'Allier en amont et aval de la confluence avec l'Artière ont montré que les apports d'eau de l'Artière pouvaient accroitre ou diluer la pollution virale de l'Allier, due aux eaux usées rejetées par plusieurs stations d'épuration en amont de la confluence. Caractérisée à 80 et 400 m de la berge, la nappe alluviale de l'Allier en aval de la confluence avec l'Artière ne présentait pas de pollution virale détectable.

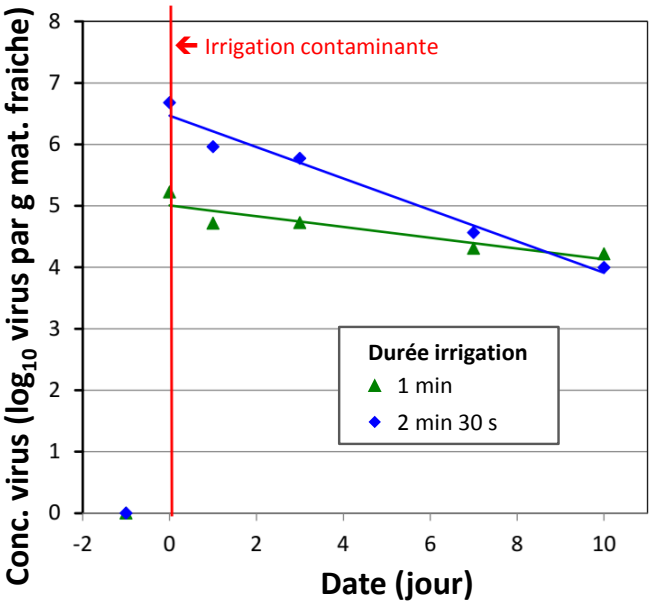
Ces résultats gagneraient à une meilleure prise en compte des apports latéraux d'eaux aux rivières (ruissellement, fossés …), des échanges d'eau entre rivière et aquifère, et du devenir même des virus en rivière.

Les eaux réutilisées en irrigation peuvent s'écouler à la surface des parties aériennes des couverts végétaux cultivés et/ou s'infiltrer dans le sol pour s'y redistribuer et être absorbées par les racines des plantes ou être entrainées vers l'aquifère sous-jacent. Les virus dans ces eaux peuvent adhérer à la surface des parties aériennes comme des parties souterraines des végétaux cultivés, tandis que les virus emportés dans le sol peuvent être internalisés dans les plantes *via* leur système racinaire et migrer ensuite vers les parties aériennes sans perdre leur pouvoir infectieux. Mais le sol peut freiner ou réduire la contamination des plantes, voire éliminer ce risque, avec l'adsorption (réversible ou non) de virus à la surface de particules de sol et/ou avec leur inactivation, voire leur destruction.

Les eaux usées traitées réutilisées par les agriculteurs de l'ASA *'Limagne Noire'* subissent un lagunage comme traitement tertiaire à la sortie de la station d'épuration. Des caractérisations des concentrations virales ont été faites à deux reprises sur les huit lagunes se succédant ; les mêmes caractérisations ont été faites sur la huitième lagune toutes les deux semaines en période de réutilisation des eaux usées. Dans cette dernière lagune, les teneurs en virus étaient quasi-systématiquement en-deçà des seuils de détection. De la même manière, aucune contamination virale n'a pu être détectée lors d'un prélèvement ponctuel d'eau usée en sortie canon d'aspersion et en surface des plants de maïs irrigués.

Des expérimentations complémentaires ont été conduites sous serre sur une culture d'oignons verts cultivés sur un sol typique de la Limagne Noire et irrigués au goutte-à-goutte selon trois modalités (enterrée, de surface, aérienne). Les eaux d'irrigation étaient contaminées à une seule date par un virus proche du norovirus de l'Homme. Elles ont montré qu'une irrigation avec des eaux chargées en virus contaminait la plante en surface, mais que les irrigations ultérieures permettaient une décontamination progressive dont la vitesse croît avec la quantité journalière d'eau reçue. S'il restait des virus en surface après quelques jours, il est très probable que ceux-ci aient été inactivés suite à leur exposition à de l'air non saturé en vapeur d'eau. Le mode d'irrigation aérien, de surface ou enterré affectait bien évidemment la contamination des tiges-feuilles, mais n'affectait pas la





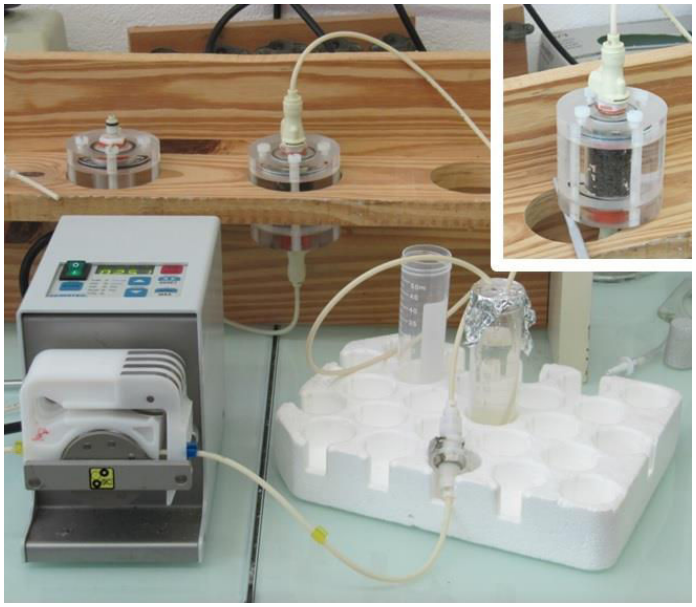
Trois modes d'irrigation d'oignons verts (Allium) sous serre avec une irrigation quotidienne et à une date unique irrigation par une solution contaminée en virus

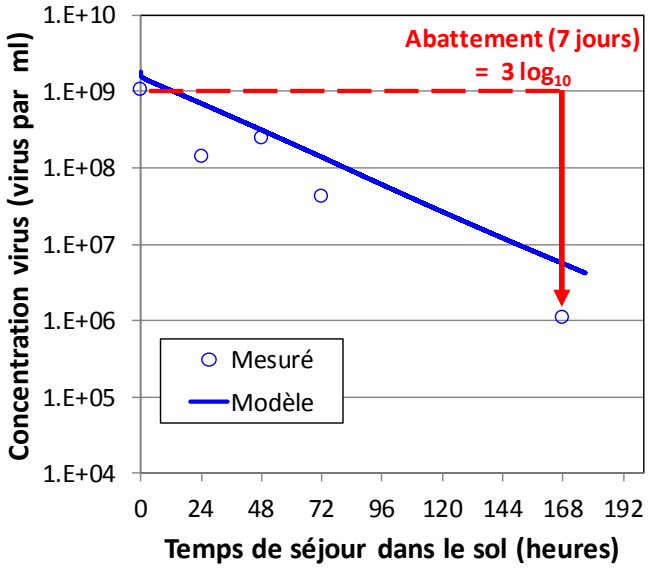
contamination de surface des bulbes.

A partir d'expérimentations sur de petites colonnes d'agrégats du sol de Limagne Noire saturées en eau, nous avons montré que les virus apportés disparaissaient progressivement de la solution circulant dans la colonne : leur concentration était divisée par 2,4 environ toutes les 24 heures. L'utilisation d'une solution non contaminée pour "rincer" ce sol ne permettait de récupérer qu'une très faible fraction des virus disparus et l'utilisation d'un protocole "plus violent" défini pour maximiser la remobilisation des virus restant adsorbés à la surface des particules de sol ne permettait de récupérer que très peu de virus en plus, suggérant la destruction de ces virus. La composition chimique de la solution traversant le sol impactait la disparition des virus ou leur remobilisation. Notamment de fortes teneurs en ion magnésium favorisaient l'immobilisation du virus ou ralentissaient sa remobilisation, comme cela a été observé avec de l'eau de nappe prélevée en Limagne Noire.

Malgré l'abattement provoqué par le sol sur la quantité de virus pouvant migrer vers les racines, on a observé une internalisation de virus dans les oignons vers avec contamination des bulbes et des tiges-feuilles. Contrairement aux contaminations de surface des tiges-feuilles dont la contamination dépend du mode d'irrigation, les contaminations internalisées des bulbes et des tiges feuilles ne varient pas avec le mode d'irrigation, probablement parce que les racines voient à peu près les mêmes quantités d'eau avec les mêmes niveaux de contaminations.

Ces travaux doivent être poursuivis pour tester la possibilité d'une décontamination passive associée à la transpiration ou à la guttation de la plante, évaluer l'impact de molécules comme les huiles essentielles sur l'inactivation des virus, et voire au-delà de la culture l'impact des opérations post-récoltes sur le risque.





Photos du dispositif utilisé pour suivre le devenir du norovirus murin (c.à.d. de souris) dans des colonnes de sol et évolution sur 7 jours de leur concentration dans la solution circulante.

Rejet ou réutilisation ? Les risques viraux *sensu stricto* inhérents aux deux scénarios très contrastés que sont les rejets d'eaux usées traitées en rivière et leur réutilisation en irrigation agricole restent à évaluer en prenant en compte leurs impacts respectifs sur les risques de contamination de l'Homme par les eaux, les aliments ou l'air. Cette évaluation peut nécessiter de subdiviser la population en sous-groupes se distinguant par leurs habitudes et leur sensibilité *a priori* aux pathogènes. Elle peut nécessiter de prendre en compte explicitement une variabilité des phénomènes, surtout pour les populations de petites tailles, avec l'utilisation de techniques de Monte Carlo pour générer une variabilité de symptômes et d'excrétions virales entre individus souffrant tous de gastroentérites d'étiologie virale.

Quelques références pour aller plus loin

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