sciensano

EPIDEMIOLOGY OF INFECTIOUS DISEASES
TRANSVERSAL ACTIVITIES IN APPLIED GENOMICS
FOODBORNE PATHOGENS

WASTEWATER-BASED EPIDEMIOLOGICAL SURVEILLANCE

Methodological appendix

R. JANSSENS • H. MALOUX • S. HANOTEAUX • M. LESENFANTS • L. VAN POELVOORDE • N. ROOSENS • B. VERHAEGEN • J. LINUSSIO • K. VAN HOORDE • S. VAN GUCHT • K. DE RIDDER • K. BLOT • V. HUTSE

.be

WHO WE ARE

Sciensano can count on more than 950 staff members who are committed to health every day.
As our name suggests, science and health are central to our mission. Sciensano's strength and uniqueness lie within the holistic and multidisciplinary approach to health. More particularly we focus on the close and indissoluble interconnection between human and animal health and their environment (the "One health" concept). By combining different research perspectives within this framework, Sciensano contributes in a unique way to everybody's health.
For this, Sciensano builds on the more than 100 years of scientific expertise.

Sciensano

Epidemiology and public health - Epidemiology of infectious diseases

Biological health risks - Transversal activities in applied genomics

Infectious diseases in humans - Foodborne pathogens

National wastewater-based epidemiological surveillance

November 2024 • Brussels • Belgium
Reference number: D/2024.14.440/27 | ISSN: 2795-7195

R. Janssens[1]
•
H. Maloux[1]
•
S. Hanoteaux[1]
•
M. Lesenfants1[1]
•
L. Van Poelvoorde[2]
•
N. Roosens[2]
•
B. Verhaegen[3]
•
J. Linussio[3]
•
K. Van Hoorde[3]
•
S. Van Gucht[3]
•
K. De Ridder[1]
•
K. Blot[1]
•
V. Hutse[1]
•

1 Sciensano, Epidemiology and public health, Epidemiology of infectious diseases, Brussels
2 Sciensano, Biological health risks, Transversal activities in applied genomics, Brussels
3 Sciensano, Infectious diseases in humans, Foodborne pathogens, Brussels

Contact: wastewater.info@sciensano.be

With the financial support of

Partners

Please cite as: R. Janssens, H. Maloux, S. Hanoteaux, M. Lesenfants, L. Van Poelvoorde, N. Roosens, B. Verhaegen, J. Linussio, K. Van Hoorde, S. Van Gucht, K. De Ridder, K. Blot, V. Hutse. Wastewater-based epidemiological surveillance - Methodological appendix. Brussels, Belgium : Sciensano ; 2024 20p. Report number: D/2024.14.440/27 ISSN 2795-7195. Available from: https://www.sciensano.be/en/biblio/wastewater-based-epidemiological-surveillance-methodological-appendix

TABLE OF CONTENTS

ABBREVIATIONS

WWTP	Wastewater treatment plant
IE	Inhabitant equivalent

INTRODUCTION

The present report provides a detailed overview of the methodological steps needed for the surveillance from the sampling to the reporting of the results.

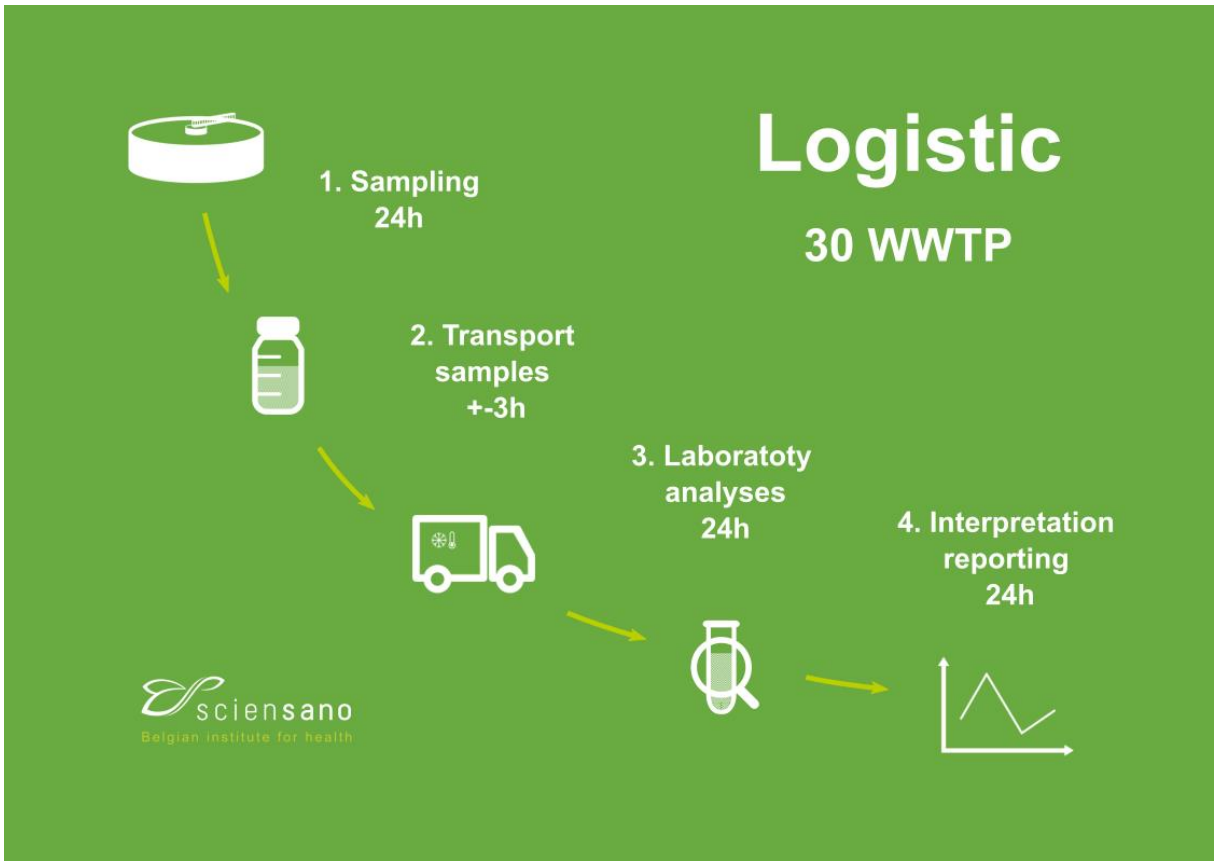


Figure 1 • Overview of the logistical and methodological steps of the surveillance, and their respective allocated time.

The surveillance of SARS-CoV-2 started in September 2020 while the surveillances of influenza and RSV started in January 2024.

Figure 2 • timeline of the key milestones since the beginning of the wastewater-based epidemiological surveillance project.

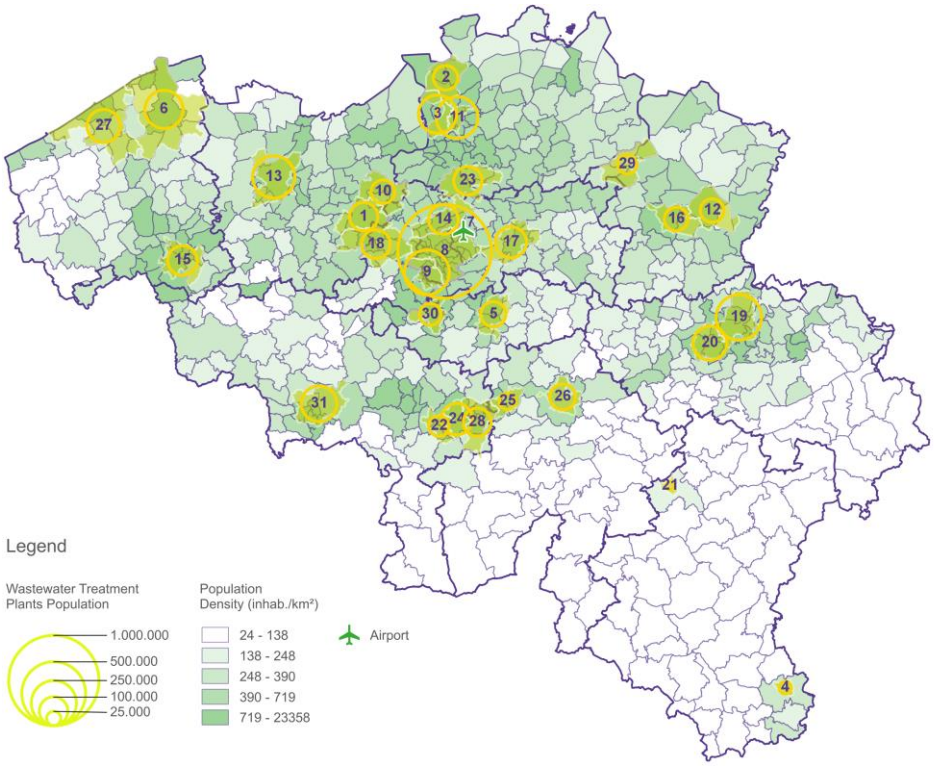
The wastewater situation can be followed through:

- The weekly bulletin on respiratory infections published in French and Dutch
- The wastewater respiratory viruses dashboard
- General information about the surveillance available on the website of Sciensano
- Data are available on the Belgian federal geoportal

METHODOLOGY

1. Samples collection

31 wastewater treatment plants (WWTPs) are included in the surveillance. Among them 30 are urban WWTPs. They can been seen in Figure 2 as well as their corresponding catchment area. A catchment area corresponds to the area for which the wastewaters of the sewage network are collected by the WWTP. Additionally, the WWTP of Brussels Airport is also included. This WWTP collects the wastewaters from the planes and the terminals.
The surveillance covers 38% of the Belgian population (cfr tables 4-6 for more details).



WWTP	ID	Population
Aalst	1	98357
Antwerpen-Noord	2	73974
Antwerpen-Zuid	3	178677
Arlon	4	19440
Basse Wavre (Dyle)	5	78290
Brugge	6	180586
Brussels Airport	7	Na
Bruxelles-Nord	8	1080145
Bruxelles-Sud	9	249307
Dendermonde	10	69500
Deurne	11	215592
Genk	12	68582
Gent	13	220130
Grimbergen	14	107643
Harelbeke	15	107894
Hasselt	16	69586
Leuven	17	120939
Liedekerke	18	101075
Liège Oupeye	19	253780
Liège Sclessin	20	142240
Marche-en-Famenne	21	8633
Marchienne-au-Pont	22	52017
Mechelen-Noord	23	98143
Montignies-sur-Sambre	24	123576
Mornimont	25	34139
Namur-Brumagne	26	82175
Oostende	27	135491
Roselies	28	96890
Tessenderlo	29	45125
Vallée du Hain (l'Orchis)	30	57826
Wasmuel	31	157343

Figure 3 • Wastewater treatment plants (WWTPs) and their catchment areas (yellow-green) included in the wastewater-based surveillance. The catchment areas of each WWTP are provided by the Vlaamse Milieumaatschappij (VMM) in Flanders, by the Société Publique de Gestion de l'Eau (SPGE) in Wallonia and by Leefmilieu Brussel/Bruxelles Environnement in Brussels. The catchment areas were redrawn in Wallonia and Brussels for a harmonized visualisation. The plane icon represents the WWTP of Brussels Airport.

The WWTPs were selected based on the following criteria:

- Plants (WWTPs) covering areas with a high population density (epidemiology-efficiency approach)
- Plants covering a large population, of more than 50.000 inhabitants (cost-efficiency approach)
- At least two plants should be present in each province
- At least 30% of the population must be covered in each region
- International entry point (e.g.: airports)

24-hour composite samples are collected once a week on Mondays from the influents of the 31 wastewater treatment plants (WWTPs). The collection is done through either volume proportional or time proportional auto-samplers. In exceptional circumstances, because of sampler failure or works (maintenance, etc.) at the treatment plant, grab samples were taken.
Once collected, the samples are stored and transported to the respective analytical laboratory, the same day (cfr figure 1), at 4°C. The samples are analysed within 24 hours. Table 1 presents the list of WWTPs with the two distinct laboratories performing the analyses.

Table 1 • List of the 31 covered WWTPs, and corresponding laboratories performing the analyses.

WWTP	Laboratory
Aalst	Sciensano
Antwerpen-Noord	University of Antwerp
Antwerpen-Zuid	University of Antwerp
Arlon	Sciensano
Basse Wavre (Dyle)	Sciensano
Brugge	Sciensano
Brussels Airport	Sciensano
Bruxelles Nord/Brussel Noord	Sciensano
Bruxelles Sud/Brussel Zuid	Sciensano
Dendermonde	University of Antwerp
Deurne	University of Antwerp
Genk	University of Antwerp
Gent	Sciensano
Grimbergen	Sciensano
Harelbeke	Sciensano
Hasselt	University of Antwerp
Leuven	Sciensano
Liedekerke	Sciensano
Liège Oupeye	Sciensano
Liège Sclessin	Sciensano
Marche-en-Famenne	Sciensano
Marchienne-au-Pont	Sciensano
Mechelen-Noord	University of Antwerp
Montignies-sur-Sambre	Sciensano
Mornimont	Sciensano
Namur-Brumagne	Sciensano
Oostende	Sciensano
Roselies	Sciensano
Tessenderlo	University of Antwerp
Vallée du Hain (l'Orchis)	Sciensano
Wasmuel	Sciensano

Sequencing for SARS-CoV-2 is performed at surges and at peaks of COVID-19 infection outbreaks. The WWTPs selected to conduct sequencing are located in the three Belgian regions: Gent, Liège Oupeye and Bruxelles Nord/Brussel Noord. Additionally, the WWTP of Brussels Airport is also included. Brussels Airport is likely an important entry point for variants on the Belgian territory.
Next-Generation sequencing is performed by the BIOTechlab at the service of Transversal Activities in applied Genomics at Sciensano.

2. Laboratory methods

The targeted sequences located in two genes of SARS-CoV-2 were selected to maximize the specificity of our analytical detection methods (specificity for all SARS-CoV-2 lineages). The N2 primers and probes target the N gene encoding for the nucleoprotein, and the E primers and probes target the E gene encoding for the viral envelope protein.

Besides SARS-COV-2, influenza viruses A and B and RSV subgroups A and B are also measured. Primers and probes for influenza viruses A and B were selected based on genomic regions highly conserved in various subtypes and genotypes. Matrix protein gene sequences were chosen for influenza virus A while hemagglutinin gene sequences were selected for influenza virus B. The N gene of RSV was chosen as the target because it is one of the most conserved genes in the RSV genome. The probes and primers are subgroup specific.

In addition to these respiratory viruses, the Pepper Mild Mottle virus (PMMoV) faecal indicator is quantified. The PMMoV is used as an indicator of the population effectively covered by the sample (through an estimation of the faecal load). See section 5 of the present report for more info on the PMMoV strategy and methodology. Details about the respective sequences can be found in table 2.

Samples are first ultrafiltrated, concentrated, and their RNA is then extracted. Analyses are done by RT-dPCR using Qiacuity (QIAGEN GmbH, Germany). The viral concentrations (copies/mL) are computed as the mean of the viral concentrations measured on the N2 and E genes for SARS-CoV-2.

Table 2 • Overview of primers/probes sequences used for the RT-dPCR assays (cc. = concentration).

Targeted gene	Primer/probe	Final cc.	Sequence (5' – 3')
SARS-CoV-2 (N gene)	nCoV_N2-F	800 nM	TTACAAACATTGGCCGCAAA
	nCoV_N2-R	800 nM	GCGCGACATTCCGAAGAA
	nCoV_N2-P	250 nM	ACAATTTGCCCCCAGCGCTTCAG
SARS-CoV-2 (E gene)	E_Sarbeco-F	800 nM	ACAGGTACGTTAATAGTTAATAGCGT
	E_Sarbeco-R	800 nM	ATATTGCAGCAGTACGCACACA
	E_Sarbeco-P	250 nM	ACACTAGCCATCCTTACTGCGCTTCG
Influenza A (M gene)	InfA-F	400 nM	GACCRATCCTGTCACCTCTGAC
	InfA-R	400 nM	AGGGCATTYTGGACAAAKCGTCTA
	InfA-P	250 nM	6-FAM/TGCAGTCCTCGCTCACTGGGCACG/ZEN/IB®FQ
Influenza B (HA gene)	InfB-F	400 nM	AAATACGGTGGATTAAATAAAAGCAA
	InfB-R	400 nM	CCAGCAATAGCTCCGAAGAAA
	InfB-P	250 nM	HEX/CACCCATATTGGGCAATTTCCTATGGC/ZEN/IB®FQ
RSV A (N gene)	RSVQA1	400 nM	GCTCTTAGCAAAGTCAAGTTGAATGA
	RSVQA2	400 nM	TGCTCCGTTGGATGGTGTATT
	RSVQA	250 nM	Cy5/ACACTCAACAAAGATCAACTTCTGTCATCCAGC/TAO/IB®RQ

RSV B (N gene)	RSVQB1	400 nM	GATGGCTCTTAGCAAAGTCAAGTTAA
	RSVQB2	400 nM	TGTCAATATTATCTCCTGTACTACGTTGAA
	RSVQB	250 nM	ROX/TGATACATTAAATAAGGATCAGCTGCTGTCATCCA/IB®RQ

Pepper Mild Mottle Virus	PMMV-R	400 nM	TTGTCGGTTGCAATGCAAGT
	PMMV-P	200 nM	CCTACCGAAGCAAATG

Sequencing is performed using the Freyja tool to obtain the SARS-CoV-2 lineages with a minimal frequency of 0.01% and depth coverage of 50. Mutations with depth coverage below 50 are excluded from the reporting.

3. Normalization using PMMoV

The normalization of the wastewater results using a faecal indicator is used to interpret results of the surveillance. The aims of this approach are to correct SARS-CoV-2 viral concentrations for:

- a possible dilution effect of concentrations measured in samples (rain events)
- the population covered in each catchment area, as well as its effective mobility

Therefore, viral to faecal ratios are computed dividing the SARS-CoV-2 viral concentrations by the PMMoV concentrations measured in samples: (SARS-CoV-2 RNA copies/mL) / (PMMoV RNA copies/mL). The PMMoV virus is a well-known indicator of human faecal contamination used to estimate the population represented by a wastewater sample. As the PMMoV and the SARS-CoV-2 are analyzed simultaneously, their ratios correct for rain dilution and population's mobility

PMMoV measures are also used as internal controls for inhibitors and dilution effects. The faecal indicator allows to highlight high dilution events leading to a viral concentration being below the LOQ, reported as a 'negative result'.

4. Viral load

In order to account for the dilution caused by rain events, quantified viral concentrations (copies/mL) are expressed as viral load per capita (SARS-CoV-2 RNA copies/day/100.000 inhabitants) by multiplying the quantified viral concentration (SARS-CoV-2 RNA gene copies/mL) by the flow (m^3) of the 24-hour sampling period and normalized with the domestic inhabitant-equivalent (IE) of the corresponding treatment plant. This method is used in parallel of the viral to faecal ratios to interpret the results of the surveillance.

When expressing the wastewater results at the national, regional, or provincial level, the means (μ) of the viral load (cci) measured in the corresponding catchment areas (i) were weighted with the domestic inhabitant equivalent (IE). Therefore, the viral loads measured in the catchment areas covering a larger population account for a larger share in the mean load computed. The following expression was used to compute the weighted mean:

$$\mu = \sum_{i=1}^{n} (cc_i * IE_i) \Big/ \sum_{i=1}^{n} (IE_i)$$

5. Wastewater-based alerting indicators

Two alerting indicators were developed to assess the wastewater-based epidemiological situation: the High Circulation and the Increasing Trend. These two indicators are computed for each covered area, on the viral to faecal ratios (cfr Section 4).
The High Circulation indicator provides information on the level of SARS-CoV-2 concentrations. The Increasing Trend indicator highlights areas where the concentrations show a rising trend for a longer term.

In further details, before computing the indicators, a normalization step is applied on the viral to faecal ratios. The normalization allows for a comparison of the viral to faecal ratios with the ones of a reference period. This way of normalizing removes the analytical bias resulting from the distinct laboratories. The normalization is performed by expressing viral to faecal ratios in percentage of the maximum value recorded during a previous viral wave for each area. It is worth mentioning that the maximum value recorded in a specific area depends on the epidemiological context, and thus varies according to the reference period considered.

In case the analytical method is different between the reference period and the moment of computing the indicators, correction factors were applied. The correction factors were computed by gene and laboratory using linear regression between the viral concentrations measured with the old and new analytical method. The correction factors are thus applied to ensure continuity and comparability of the indicators.

The format of the indicators is Boolean. Therefore, if the normalized viral to faecal ratio exceeds 30% of the highest value recorded during a virus wave of reference, the value of the High Circulation indicator is set to 1. Else it is set to 0. Additionally, the Increasing Trend is set to 1 if the moving average on the past 14 days of the normalized viral to faecal ratios has increased for 14 days or more. Otherwise, it is set to 0.

6. Population coverage

The domestic equivalent inhabitants (EI) are used to estimate the population connected to the sewage system of a WWTP. The domestic EI are based on several data sources (population, building type, network connections, and WWTPs geographical coverage areas). These domestic inhabitants equivalent are available by municipality and wastewater treatment plant.
It is worth mentioning that some municipalities are covered by several treatment plants and a treatment plant catchment area can cover more than one province or region.
The inhabitant equivalent data are provided by the Société Publique de Gestion de l'Eau (SPGE) in Wallonia and by the Vlaamse Milieumaatschappij (VMM) in Flanders. In Brussels, the population is estimated based on the population by statistical sector data provided by STATBEL and the global connection percentage to the sewage network in the region. Table 3 presents the estimated population covered in each wastewater catchment area. Tables 4, 5 and 6 present the domestic inhabitant equivalent and the resulting population coverage at provincial, regional and country levels.

Table 3 • Location of the WWTPs covered by the surveillance with their respective inhabitant equivalents (Data 2020).

WWTP	Inhabitant equivalent	Province	Region
Aalst	98 357	Oost-Vlaanderen	Flanders
Antwerpen-Noord	73 974	Antwerpen	Flanders
Antwerpen-Zuid	178 677	Antwerpen	Flanders
Arlon	19 440	Luxembourg	Wallonia
Basse Wavre (Dyle)	78 290	Brabant Wallon	Wallonia
Brugge	180 586	West-Vlaanderen	Flanders
Brussels Airport	NA	Vlaams-Brabant	Flanders
Bruxelles Nord/Brussel Noord	1 080 145	Brussels	Brussels
Bruxelles Sud/Brussel Zuid	249 307	Brussels	Brussels
Dendermonde	69 500	Oost-Vlaanderen	Flanders
Deurne	215 592	Antwerpen	Flanders
Genk	68 582	Limburg	Flanders
Gent	220 130	Oost-Vlaanderen	Flanders
Grimbergen	107 643	Vlaams-Brabant	Flanders
Harelbeke	107 894	West-Vlaanderen	Flanders
Hasselt	69 586	Limburg	Flanders
Leuven	120 939	Vlaams-Brabant	Flanders
Liedekerke	101 075	Vlaams-Brabant	Flanders
Liège Oupeye	253 780	Liège	Wallonia
Liège Sclessin	142 240	Liège	Wallonia
Marche-en-Famenne	8 633	Luxembourg	Wallonia
Marchienne-au-Pont	52 017	Hainaut	Wallonia
Mechelen-Noord	98 143	Antwerpen	Flanders
Montignies-sur-Sambre	123 576	Hainaut	Wallonia

Mornimont	34 139	Namur	Wallonia
Namur-Brumagne	82 175	Namur	Wallonia
Oostende	135 491	West-Vlaanderen	Flanders
Roselies	96 890	Hainaut	Wallonia
Tessenderlo	45 125	Limburg	Flanders
Vallée du Hain (l'Orchis)	57 826	Brabant Wallon	Wallonia
Wasmuel	157 343	Hainaut	Wallonia

Table 4 • Inhabitant equivalent and population coverage (%) at the provincial level (Data 2020).

Province	Inhabitant equivalent	Population coverage
Antwerpen	564 854	30.2%
Brabant Wallon	135 340	33.3%
Brussels	1 212 163	99.5%
Hainaut	420 555	31.2%
Liège	396 020	35.7%
Limburg	182 851	20.8%
Luxembourg	28 073	9.8%
Namur	125 585	25.3%
Oost-Vlaanderen	409 093	26.8%
Vlaams-Brabant	428 590	37.1%
West-Vlaanderen	423 971	35.3%

Table 5 • Inhabitant equivalent and population coverage (%) at the regional level (Data 2020).

Region	Inhabitant equivalent	Population coverage
Brussels	1 212 163	99.5%
Flanders	2 009 359	30.3%
Wallonia	1 105 573	30.3%

Table 6 • Inhabitant equivalent and population coverage (%) at the country level (Data 2020).

Country	Inhabitant equivalent	Population coverage
Belgium	4 327 095	37.7%

7. Data management and analysis

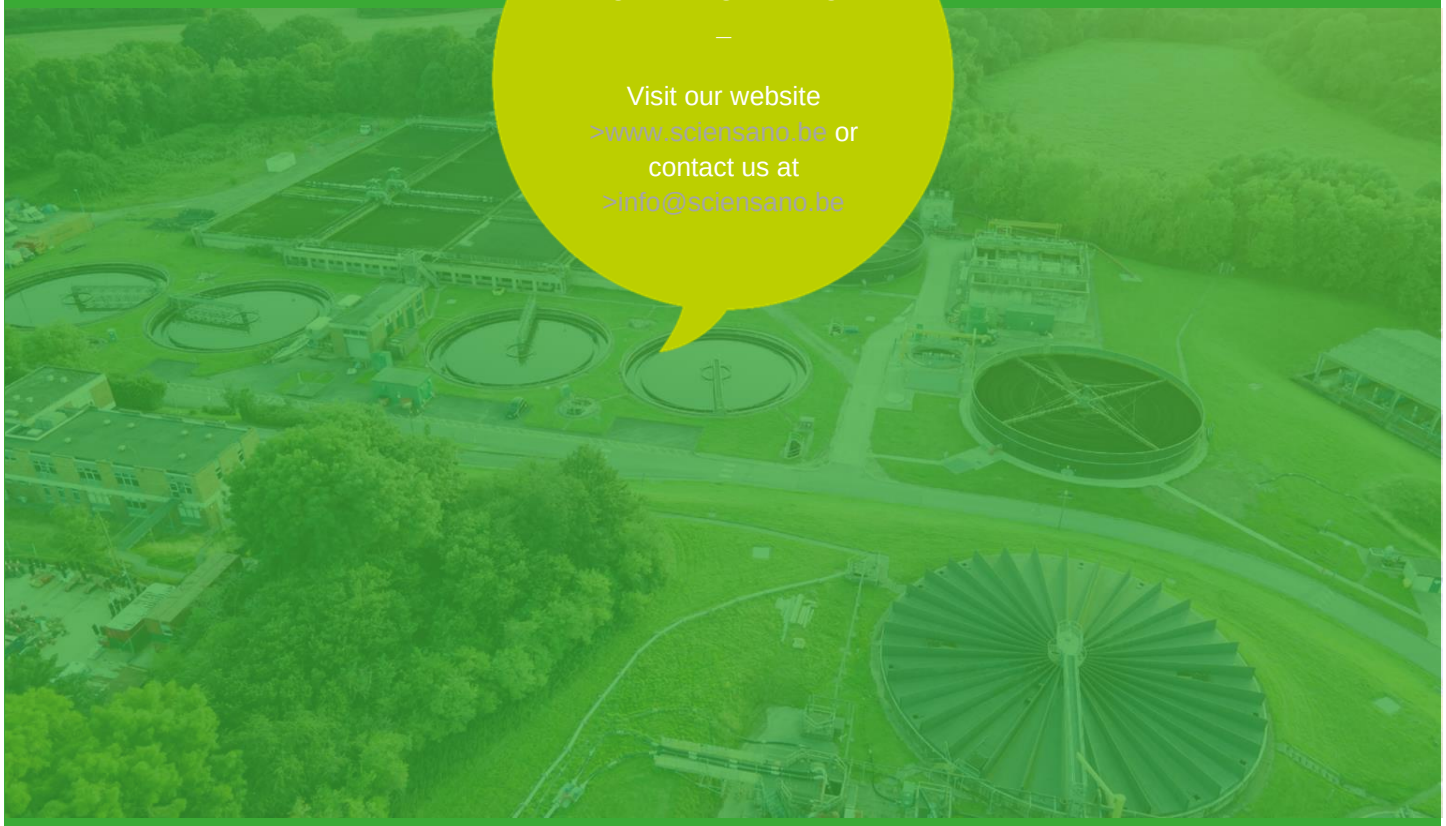
Data management was performed using SAS (SAS 7.15, NC, USA) while statistical analysis and visualization were carried out with the R software (version 4.2.3).

CONTACT
wastewater.info@sciensano.be

MORE INFORMATION

–

Visit our website
>www.sciensano.be or
contact us at
>info@sciensano.be



Sciensano • Rue Juliette Wytsmanstraat 14 • Brussels • Belgium • T + 32 2 642 51 11 • T press + 32 2 642 54 20 • info@sciensano.be • www.sciensano.be

Responsible editor: C. Léonard, Managing director • Rue Juliette Wytsmanstraat 14 • Brussels • Belgium • D/2024.14.440/27 • ISSN 2795-7195