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Monitoring waves of the COVID-19 pandemic: Inferences from WWTPs of different sizes

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HIGHLIGHTS
• SARS-CoV-2 loads correlate much better than concentrations with infection incidence.
• Higher SARS-CoV-2 loads were measured during the first wave than the second wave.
• Large WWTPs have quantifiable levels at lower infection incidence than small WWTPs.
• Monitoring of small WWTPs is challenging and depends on high COVID-19 incidence.

GRAPHICAL ABSTRACT

ABSTRACT

Wastewater based epidemiology was employed to track the spread of SARS-CoV-2 within the sewershed areas of 10 wastewater treatment plants (WWTPs) in Catalonia, Spain. A total of 185 WWTPs inflow samples were collected over the period consisting of both the first wave (mid-March to June) and the second wave (July to November). Concentrations of SARS-CoV-2 RNA (N1 and N2 assays) were quantified in these wastewaters as well as those of Human adenoviruses (HAdV) and JC polyomavirus (JCPyV), as indicators of human faecal contamination. SARS-CoV-2 N gene daily loads strongly correlated with the number of cases diagnosed one week after sampling i.e. wastewater levels were a good predictor of cases to be diagnosed in the immediate future. The conditions present at small WWTPs relative to larger WWTPs influence the ability to follow the pandemic. Small WWTPs (<24,000 inhabitants) had lower median loads of SARS-CoV-2 despite similar incidence of infection within the municipalities served by the different WWTP (but not lower loads of HAdV and JCPyV). The lowest incidence resulting in quantifiable SARS-CoV-2 concentration in wastewater differed between WWTP sizes, being 0.11 and 0.82 cases/1000 inhabitants for the large and small sized WWTP respectively.

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1. Introduction

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), went from a local issue in China to a truly global threat to human health, wellbeing and the
economy, all in a matter of a few months. As of the end of November 2020, the disease has caused over 64 M infections and 1.4 M deaths and has dictated the agenda of governments for the past months (WHO, 2020).

The dominant impact the pandemic has had on everyday life has also resulted in a scientific and technological scramble for solutions, amongst which has been the application of wastewater-based epidemiology (WBE) to track the distribution and magnitude of infections amongst the population generating the wastewater (WW) (Medema et al., 2020a, 2020b; Mallapaty, 2020; Lesté-Lasserre, 2020; Ahmed et al., 2020a; Randazzo et al., 2020). While COVID-19 is mainly a respiratory illness, SARS-CoV-2 can also infect intestinal cells and it is shed in faeces in moderate quantities (between $10^5$ and $10^7$ GC/g) (Cheung et al., 2020; Wolffel et al., 2020; Zheng et al., 2020). This has prompted suggestions to apply WBE principles in tracking the spread of the disease (Bivins et al., 2020; Daughton, 2020; Hata and Honda, 2020), with the first reports of detection and quantification of the virus in wastewater (WW) in April 2020 (Ahmed et al., 2020a; Lodder and de Roda Husman, 2020; Medema et al., 2020b).

The theoretical scientific basis for tracking the virus in WW has been previously described (see Ahmed et al., 2020a; Wu et al., 2020), however, a number of practical uncertainties still persist. While these issues hinder the application of WBE for accurately measuring the actual number of infected individuals, the potential advantages – that an approximation affords to public health monitoring – are too valuable to dismiss. Being able to obtain an estimate of the degree of active cases within a specific community is paramount to controlling the spread and obtaining this information through WBE is cheaper and less laborious than carrying out diagnostic screening of individuals at a massive scale. At the initial stages of the pandemic, the health authorities in most countries struggled to cope with the sudden influx of testing requirements. As a consequence of this, the number of individual nasopharyngeal or oral swabs carried out during the first wave of the pandemic to determine if suspected cases were in fact positive had to be prioritised. Thus, the real degree of infection (actual prevalence) amongst a community could not be established. This was also later confirmed by seroprevalence studies in numerous countries which showed that a much larger portion of the populations under study were positive to SARS-CoV-2 antibodies, indicating that they have been or are infected with the virus (Eckerle and Meyer, 2020; Pollán et al., 2020). In clear contrast, the WBE approach bypasses the limitations of individual testing since it provides integrated information of the whole community. A few WW samples can provide the same population-level information as thousands of individual tests. Clearly WBE is not mean to replace individual testing controls before any processing was carried out. All samples were spiked with the bacteriophage MS2 as process control as well as to identify any dilution or other undesired mixing of WW. All this information is expected to aid the application of WBE in decision making by better defining the expectations and limits of the approach to policy makers.

2. Materials and methods

2.1. Wastewater samples – viral quantification

A total of 185 influent 24 h time-proportional composite WW samples from mid-March till early–November 2020 from 10 WWTPs, of varying sizes, in the Region of Catalonia (Spain) were collected. All samples were collected as a 24-h composite using the autosamplers available at the WW facilities. The monitored WWTPs (Table 1) serve 26% of the Catalan population (2.0 M people of a total of 7.6 M), including more than half of the City of Barcelona, one of the most COVID-19 affected areas in Spain with almost 300,000 cases by the end of November. All samples (250 mL) were collected at the studied WWTPs using gloves and sterile containers, transported at 4 °C and delivered to the laboratory at The University of Barcelona and stored at —80 °C until analysis.

As back up during analysis, 150 mL of each wastewater sample was stored. Concentration of the viral particles was performed by first removing debris by centrifuging 100 mL of sample at 4750 ×g for 30 min and processing the supernatant for concentration by ultrafiltration. Due to the severe shortage of filtration devices caused by the COVID-19 pandemic, concentration of viruses was initially achieved using Centricon® Plus-70 30 KDa (Millipore) and from 15th May onwards the automatic Concentrating Pipette (CP-Select™) with 150 KDa ultrafiltration tips (Innovaprep) was used. A recent study characterizing both Centricon® devices and the new CP-Select™ have shown that both concentration methods yield equivalent results (Forés et al., 2021). When using Centricon®, the viral particles present in a volume of 70 mL of supernatant were concentrated in 200–250 μL as previously described (Medema et al., 2020a). When using CP-Select™, between 80 and 90 μL of supernatant were filtered and eluted in a final volume of 240–300 μL. The concentration factors were thus between 280–350 × for the Centricon® and between 266–375 × for the CP-Select™. All samples were spiked with the bacteriophage MS2 as process control before any processing was carried out.

Viral DNA and RNA were co-extracted with QIAamp Viral RNA Mini Kit using the QIAcube automatic system (Qiagen). A total of 140 μL of sample concentrates were extracted into a final volume of 60 μL. To determine the genome copy numbers of SARS-CoV-2, the N1 and N2 assays targeting the viral nucleocapsid (US CDC, 2020) were used. Specific qPCR and RT-qPCR assays previously described were used to quantify MS2 bacteriophage (Pecson et al., 2009), HAdV (Boffill-Mas et al., 2006; Hernroth et al., 2002) and JCPyV (Pal et al., 2006) by using TaqMan™ Environmental Master Mix 2.0 and RNA UltraSense™ One-Step RT-qPCR System (Invitrogen) for DNA and RNA viruses respectively. Primers, probes and qPCR conditions are summarized in Table 1 of the supplementary material. The SARS-CoV-2 standard was...
The overall mean viral recovery of the applied procedure using MS2 as a process control was 30.19% (with a 95% interval of confidence between 27.10 and 33.29%). Ultrafiltration with centrifugal ultrafilters that have been recently used for the concentration of SARS-CoV-2 from wastewater, reported similar results with MS2 as the process control (Forés et al., 2021; Medema et al., 2020a, b). Although the physicochemical parameters between WWTPs were significantly different (see boxplots SI Fig. 1), no significant differences were observed amongst the viral recoveries (p-value = 0.069).

3.2. SARS-CoV-2 loads over the first and second waves

The WWTPs – WWTP-J (population served: 1,497,767), WWTP-H (population served: 93,796) and WWTP-G (population served: 66,048) were monitored for SARS-CoV-2 abundance (N1 and N2 assay) starting from mid-March and results till the start of November. Fig. 1 shows the SARS-CoV-2 quantification (N1 assay) and the estimates of cases as per Eqs. (2)–(3). Background coloured areas indicate the different containment measures imposed on the respective
The highest concentration of SARS-CoV-2 in WW was obtained for WWTP-J in Barcelona, which reported infections just 4 days later (26th of March, 556 new daily cases). The anticipation is also seeing through the correlations shown in Fig. 2.

The highest concentration of SARS-CoV-2 in WW was observed on the 28th of March in Barcelona, which reported the highest number of daily new cases. In WWTP-J, the highest N1 concentrations in WW was recorded on the 8th of April while the peak of daily new cases was reported on the 29th of March and the peak for the cumulative 7 day sum was two days later (31st of March). Less clear is the N1 peak observed a week later (8th of April) and the lack of detection in the following week (16th April) since no dilution by rainfall applied.

Besides, the concentration of other viral indicators (HAdV and JCPyV) in this sample was similar to preceding and following dates (SI Table 2). The concentration of other viral indicators (HAdV and JCPyV) in this sample was similar to preceding and following dates (SI Table 2).

The efficacy of the lockdown measures implemented nationwide are well reflected in the results from WW monitoring. Spain went into a nationwide lockdown on the 14th of March although some hotspot areas were locked down earlier (such as the city served by WWTP-G). This lockdown was organised in four phases of incremental restrictions, being the phase 0 the most restrictive and phase 4 the least. During the lockdown (orange shading in Fig. 1a–c), the concentration of SARS-CoV-2 in sewage progressively decreased together with daily new cases and estimates of infection prevalence. The lockdown in Spain was highly restrictive and generally well enforced, allowing only the bare essential movements. SARS-CoV-2 concentrations in sewage were reduced to below LOD in subsequent phases (Fig. 1a–c green shading) agreeing with the low number of reported cases during this period. The following phases, which mandated social distancing, mask wearing and limited some social activities while allowing travel between regions (Fig. 1a–c grey shading) quickly resulted in an increase of N1 concentrations above the LOD. Similar observations were reported in France after the lockdown and where concentrations of SARS-CoV-2 in sewage increased by 1.5 orders of magnitude after relaxing the restrictions (Trottier et al., 2020). The anticipation of the infection peak through WW is not as evident for the second wave, however the correlations done in Fig. 2 still show the best fit with the number of cases for the following 7 days of sampling. Overall, the health authorities and population were better prepared for the second wave and the lag in identifying cases was probably shorter and hence not as evident as for the first wave in Fig. 1.

The WW concentrations of SARS-CoV-2 RNA measured herein are well within the range of reported values in other WBE SARS-CoV-2 studies. Medema et al. (2020b) reported first wave maximum concentration of 7.9 × 10^5 GC/L (N1) in the Tilburg, Netherlands on the 16th of March (compare to. 3.9 × 10^6 GC/L recorded on the 22nd of March in Barcelona which saw a higher incidence of infection than the Netherlands). Analysis of SARS-CoV-2 in wastewater collected from other Spanish WWTPs located in areas with similar COVID-19 incidence during April (1 case per 1000 inhabitants) reported concentrations (N1 and N2 assays) ranging between 10^5 and 10^6 GC/L. Randazzo et al. (2020). In Germany, WW concentrations reported for April (Westhaus...
et al., 2021) were up to 2 orders of magnitude lower than values reported herein despite having similar reported incidence rates (0.5–1.8 cases per 1000 inhabitants). Germany as a whole suffered a more contained COVID-19 outbreak during the first wave and the mismatch could be due to a higher detection rate of COVID-19 cases in Germany relative to Spain.

During the first wave in Catalonia, PCR tests were less readily available giving estimates that under-reported cases (Russell et al., 2020). In fact, it has been documented that the median viral loads in clinical samples over the first phase of the outbreak were higher as compared to the following period (Jacot et al., 2020). The higher WW loads during the first wave reinforces the idea that the number of real infections in the first wave was higher than the second wave, despite the fact that more cases were diagnosed during the second wave i.e. the percentage of cases identified out of the total cases was higher in the second wave.

3.3. Relationship between SARS-CoV-2 loads and COVID-19 incidence

Fig. 2 shows the linear correlation between either the log_{10} converted values of absolute concentration (in GC/L, blue dots, left y-axis) or total viral load (in GC/d, orange dots, right y-axis) and the estimation of COVID-19 prevalence for all three cities in the studied period (March to September). The best fit, in terms of the highest Pearson correlation coefficient and lowest RSME is seen from the regression of log daily viral loads (Fig. 2a & d-orange) against the summation of cases in the 7-days that followed WW sampling (Eq. (3)). That is the viral loads in WW are a better indicator of the cases that will be diagnosed within 1 week after sampling thus affording a level of anticipation of future values. The worst fit was observed with estimates of viral incidence that summed the number of new cases in the 7-days that preceded the sampling (Fig. 2c & f). Comparing the fit parameters, for the same estimation of cases, between waves, shows that the second wave data were better than that of the first wave. This is probably a result of health authorities being able to diagnose a larger proportion of the real number of cases during the second wave as opposed to the first in March–June.

3.4. Inferences from WWTPs of different sizes

From the beginning of the second wave (second peak of COVID-19 infections), a total of 10 WWTPs, the three already shown, and an additional seven were added to the monitoring campaign. This new dataset included a wider range of WWTPs sizes (the largest of which are WWTP-J and WWTP-I (Table 1) and different geographical areas in Catalonia. The large and medium sized WWTPs showed a similar increasing trend in the concentrations of SARS-CoV-2 (N1 assay) from July to November coinciding with the progressive rise of reported cases during this period i.e. the so called second wave (Fig. 3). Significant levels of human faecal contamination, by means of JCPyV quantification, were persistently detected over the sampling period in all tested facilities, indicating that the non-detected or low SARS-CoV-2 values were not due to the presence of PCR inhibitory materials in the WW. The recovery of the bacteriophage MS2 also confirmed that there was no failure in the analytical process (mean calculated recovery values were 40.50 ± 22.88%).

Besides the incidence level, concentrations of SARS-CoV-2 (N1 and N2) were consistently higher in larger facilities compared with smallest plants (Fig. 4). This trend was not observed for the human faecal indicators analysed (HAdV and JCPyV), showing no differences according the
size of the population served. Like SARS-CoV-2, some infections of the \( \text{HAdV} \) virus cause gastrointestinal and respiratory diseases (Rusiñol and Girones, 2017). Respiratory adenoviruses are detected in nasopharyngeal swabs at lower mean viral loads \( (5.0 \times 10^3 \text{ GC/mL}) \) than SARS-CoV-2 \( (6.0 \times 10^6 \text{ GC/mL}) \) (Jacot et al., 2020). Although the level of excretion in faeces is relatively similar between the viruses \( (\log_{10} 7–10 \text{ GC/g}) \) (reviewed by Rusiñol and Girones, 2017 and Medema et al., 2020a, b), the detection of SARS-CoV-2 RNA in stool samples is limited to a shorter duration of shedding (from 1 to 33 days; (Zheng et al., 2020)) compared to \( \text{HAdV} \) (up to 192 day after infection (Lion et al., 2010)). Polyomaviruses, and specially 
\( \text{JCPyV} \), are also persistently excreted by infected individuals with or without symptoms of a disease, sometimes for years, and in this case via the urinary tract (Bofill-Mas, 2016; Moens et al., 2013). The excreted viral particles found in wastewater will thus

Fig. 3. Estimated number of active infections (lines, right y-axis) and WW loads of SARS-CoV-2 (N1 assay), orange bar plot left y-axis) and \( \text{JCPyV} \) (blue bar plot left y-axis) viruses for the second wave at all 10 WWTPs.
originate from faeces, urine and also oral fluids and their persistence in this complex water matrix will determine the chance of detection. It has been calculated that 90% of the viable SARS-CoV-2 \( (T_{90}) \) are inactivated in 1.5 days in wastewater at room temperature (Bivins et al., 2020) whereas, using qPCR and DNase treatment to quantify structured viral particles, the \( T_{90} \) of HAdV is 60.9 d and that of JCPyV is 63.9 d (Bofill-Mas et al., 2006). Previous studies on small WWTP (approximately 2100 inhabitants) have validated the use of both faecal markers, JCPyV and HAdV, as they are not affected by day-to-day variations (Mayer et al., 2016). HAdV and JCPyV have been described to occur at much more similar concentrations in WW (Bofill-Mas et al., 2006; Rusiñol and Girones, 2017) than in the current study in which JCPyV concentrations are higher. A decrease in HAdV concentrations were observed since March 2020 (SI Table 2) thus it might be that lockdown avoided community transmission of HAdV while the transmission of JCPyV, that has been described to occur within families, could have not been affected.

During the first weeks of the second wave, no SARS-CoV-2 RNA was detected in the smallest WW facilities (WWTP-A, —B, —C and —D), it was not until mid-September, when the infection rate relative to population was higher than ever (including the first wave), that SARS-CoV-2 was detected and quantified in their WW samples. In terms of population served these four WWTPs are the smallest of the WWTP studied herein and all serve less than 27,000 inhabitants (Table 1). Since the concentration of HAdV and JCPyV in these WWTPs were similar to those measured in the other WWTPs, the lack of detection of SARS-CoV-2 in WW was not caused by dilution of WW from other sources such as industry or storm water, nor by failures in the analytical process. This substantiates our working hypothesis that small WWTPs are not as informative to sample as their larger counterparts since a higher proportion of infection in needed within the sewershed in order to produce quantifiable WW results. A summary of the results from the sampling carried out during the second wave are shown in Fig. 4. The JCPyV and HAdV viruses were quantifiable in most samples independently of WWTTs size and the concentration was very similar in between WWTPs, this was in contrast with the N1 and N2 assays. Correlation analyses were performed between the mean concentrations and the maximum incidences reported. Bigger WW facilities presented in mean values higher incidences (Pearson’s correlation coefficient p-value = 0.85) (see Fig. 2 Supplementary material). Other authors have observed positive correlation between the SARS-CoV-2 concentrations and the active cases reported even where low COVID-19 prevalence was reported (D’Aoust et al., 2021; Medema et al., 2020a, b).

Although SARS-CoV-2 RNA can be detected in WW from small facilities when only few clinical cases are reported (Gonzalez et al., 2020), sporadic detection would not allow consistent interpretation. The difference in prevalence of infection and WWTP size is also visualised in Fig. 5. While all but one sampling dates recorded a prevalence rate higher than 0.1 cases per 1000 inhabitants, small WWTPs were more likely to have WW levels lower than the LOD. The samples from the large WWTP-J were quantifiable (i.e. above LOD) 100% of the time, the lowest WW quantifiable incidence (LWQI) for this plant was recorded at 0.59 cases per 1000 inhabitants. A lower incidence was never recorded thus it is highly possible that WW level would remain...
positive even at lower incidences. A stark difference in the LWQI is seen between the small (LWQI = 0.82 cases/1000 inh.) and medium (LQWI = 0.11 cases/1000 inh.) sized WWTPs.

In other words, while the analytical LOD in terms of GC/L are the same amongst samples from different WWTPs, the LOD in terms of prevalence of infection (i.e. incidence of shedding relative to total number of inhabitants) is higher in small cities. It is well documented that obtaining a representative WW sample from a small population is more challenging than for large populations (Aymerich et al., 2017; Medema et al., 2020a, b; Ort et al., 2010). Putting incidence between WWTPs into perspective, and incidence rate of 0.7 cases/1000 inh. is only between 5 and 19 cases for the four smallest WWTPs (WWTP A-D) but over 1000 in the largest WWTP. The variability with such small numbers is high; for example, a portion of the infected individuals might never shed the virus in faeces throughout the course of their infection, some might be residing in other municipalities while being registered in a different one. Another factor is the high variability in viral shedding both in terms of loads and duration which would be even more extreme with such low numbers of active cases since they would not average out. One has also to bear in mind that toilet use and flushing occurs in discrete events of a short duration. A composite WW sample that is collected through pulses, tens of minutes apart, may very well miss a relevant discharge from a shedding individual. The sewer residence time, sewage fluid dynamics and composition are also expected to be influential on the sampling variability and hence utility of using WBE to monitor small areas with a low number of infected individuals in absolute terms.

Sampling the septic tanks of cruise ships and aeroplanes for SARS-CoV-2 RNA has already been carried out and positive samples were identified (Ahmed et al., 2020b). While cruise ships and aeroplanes might seem superficially similar to very small cities or neighbourhoods, the conditions for WW are very different. In aeroplanes, for instance, WW is often not diluted during flushing since the toilets are vacuum driven and greywater entry, and hence dilution, is limited to hand washing. Septic tanks on an aeroplane also allows for a better homogenisation of WW resulting in sampling strategy being less critical and grab samples being sufficiently representative. When it comes to the large WWTP-J, 0.7 cases per 1000 inhabitants corresponds to 1048 cases in absolute terms. In this case, even with the same sampling uncertainties, the probability of sampling during a relevant discharge event is much higher and the distribution of discharge is more homogeneous over time. While it is more challenging to sample small WWTPs with low incidence of infection, it is not excluded that if the practical limitations of representative sampling and homogenisation are overcome, small WWTPs could also be as useful to sample as their larger counterparts.

The three largest incidence levels which had WW values at or below LOD were reported at, 6.4 cases per 1000 inhabitants (WWTP-B, 52 cases), 3.2 cases/1000 inh. (WWTP-G, 211 cases) and 2.0 cases/1000 inh. (WWTP-F, 116 cases). Hart and Halden (2020) modelled a range of minimum infection rates needed in order to surpass the LOD. Depending on a range on numerous parameters they approximate that the upper bounds are 0.00005% and the lower bounds at 0.88%. While our results fall within this range, they are at the lower end i.e. higher prevalence of infection is need in order to be able to measure WW concentrations above LOD. The concentration and loads for small plants are rather variable, as an example, on the 29th of October 2020 an incidence of 0.324% (23 cases) was recorded in the area served by WWTP-A. This gave a concentration of 4.39 × 10^4 GC/L (daily flow = 1163 m³) while a virtually identical incidence of 0.329% (87 cases) in WWTP-D gave a concentration of 5.31 × 10^4 GC/L (daily flow =4421 m³), note that WWTP-D serves 3.7× more people than WWTP-A.

Interestingly, positive samples have been detected when reported COVID-19 cases reached 0.05–0.10 cases per 1000 inhabitants (0.005–0.010%) (Hata et al., 2020). The same authors calculated that SARS-CoV-2 was detectable if one in 100,000 persons shed 10^9 GC/g of faeces, which has been later reported to occur only in a low percentage of the population (≈10%) (Endo et al., 2020). The presence of a patient with such a high load may be possible, because shedding rates are highly variable amongst individuals (reviewed by (Medema et al., 2020a, b)) but the presence of this “superexcretor” in the smallest WWTP will be less probable. One should also keep in mind that such comparisons between different WWTPs and, especially, between different countries are error-prone since they might have different ratios of identified cases to total real cases, the latter of which can only be approximated by epidemiological models.

Overall, the higher incidence prevalence required within a population served by small WWTPs does not mean that these plants should not be monitored but the limitations in terms of sensitivity should be kept in mind, and great care should be taken when comparing WWTPs of difference size. Alternative sampling strategies could also be employed such as primary sludge sampling. Promising results have been reported (Balboa et al., 2020; Peccia et al., 2020). Limitations with this approach include that sampling proportionally at a city or
neighbourhood level (as opposed to sampling from WWTPs that are designed to precipitate the sludge) is not practical and issues with homogenisation of the sample are expected to increase uncertainty.

4. Conclusions

Applying WBE to track the spread of COVID-19 within the sewershed areas served by small WWTPs (<24,000 inhabitants) is more challenging. Higher prevalence of infection amongst the population is required in these areas in order to have WW level of SARS-CoV-2 (N1 and N2 assays) above the LOD.

- Wastewater SARS-CoV-2 GC (N1 assay) in terms of total loads is a much better predictor of prevalence of infection than SARS-CoV-2 GC concentration.
- In all 10 of the monitored WWTPs, WW loads are congruent with estimations of active cases within the sewershed.
- Concentrations of SARS-CoV-2 (N1 assay) in WW showed good correlations with the number of cases that would be diagnosed from 0 to 7 days after sampling.
- When lack of SARS-CoV-2 RNA detection, HAdV and JCPyV surveillance can help understanding dilution effects or failures in the analytical process.
- The lowest incidence of infection which resulted in a quantifiable concentration of SARS-CoV-2 in WW for medium sized WWTPs was 0.11 cases/1000 inh. while for small WWTPs this was 0.82 cases/1000 inh.

CRediT authorship contribution statement

Rusíoľ M.: sampling, sample analysis, data gathering, data analysis and writing.
Zammit I.: sampling, data gathering, data analysis and writing.
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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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