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Short Communication

Cold-chain free nucleic acid preservation using porous super-absorbent polymer (PSAP) beads to facilitate wastewater surveillance

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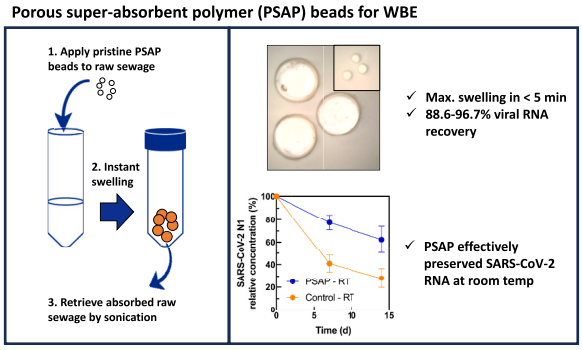
HIGHLIGHTS

- Instability of RNA virus in sewage is a challenge in managing wastewater monitoring in remote areas.
- Porous superabsorbent polymer (PSAP) beads were applied to absorb raw sewage.
- SARS-CoV-2 RNA decrease in PSAP beads was lower than control after 14-day room temperature incubation.
- PSAP beads demonstrated effectiveness in preserving viral RNA in sewage.

GRAPHICAL ABSTRACT



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ABSTRACT

The instability of viral targets including SARS-CoV-2 in sewage is an important challenge in wastewater monitoring projects. The unrecognized interruptions in the 'cold-chain' transport from the sample collection to RNA quantification in the laboratory may undermine the accurate quantification of the virus. In this study, bovine serum albumin (BSA)-modified porous superabsorbent polymer (PSAP) beads were applied to absorb raw sewage samples as a simple method for viral RNA preservation. The preservation efficiency for SARS-CoV-2 and pepper mild mottle virus (PMMoV) RNA were examined during storage for 14 days at 4 °C or room temperature against the control (no beads applied). While a non-significant difference was observed at 4 °C (~80 % retention for both control and PSAP-treated sewage), the reduction of SARS-CoV-2 RNA concentrations was significantly lower in sewage retrieved from PSAP beads (25–40 % reduction) compared to control (>60 % reduction) at room temperature. On the other hand, the recovery of PMMoV, known for its high persistence in raw sewage, from PSAP beads or controls were consistently above 85 %, regardless of the storage temperature. Our results demonstrate the applicability of PSAP beads to wastewater-based epidemiology (WBE) projects for preservation of SARS-CoV-2 RNA in sewage, especially in remote settings with no refrigeration capabilities.

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1. Introduction

Wastewater-based epidemiology (WBE) has been recently applied to detect the spread of SARS-CoV-2 RNA in various settings and communities. This approach can identify the virus in stool or sewage samples from individuals, whether they display COVID-19 symptoms or are asymptomatic (Wu et al., 2020; Zhang et al., 2020). Thus, WBE can pinpoint areas where clinical testing can be most effective by detecting viral shedding in sewer flow (Miyani et al., 2020; Cha et al., 2023). This can be especially valuable in remote regions with limited access to clinical testing and/or poor settings, and thus facilitate the efficient tracking of the disease and containment strategies (D'Aoust et al., 2021).

One important challenge in managing wastewater monitoring in remote areas is the instability of SARS-CoV-2, or other similar RNA virus targets, in sewage. SARS-CoV-2 RNA in sewage is reported to decay within hours to days (Bivins et al., 2020; Baldovin et al., 2021; Wurtzer et al., 2021). Consequently, the measured SARS-CoV-2 concentrations in wastewater may not fully represent the prevalence of the disease in the monitored community if time between sampling and RNA quantification in the laboratory is longer than a couple hours. Further, the heterogeneous nature of the sewage matrix can contribute to the degradation of the RNA (Bayati et al., 2022). In order to minimize the loss of signal during the transit, "cold-chain" is necessary to be maintained (Li et al., 2021) as refrigeration is effective in slowing down the degradation of RNA or intact viral particles in wastewater samples. Any disruptions in the cold-chain introduced from the on-site sample collection to the RNA quantification in the laboratory is essentially an unknown variable in practice, which can compromise the precise estimation of the level of virus in sewer systems. Therefore, it is highly important to establish reliable and simple methods to preserve SARS-CoV-2 RNA in wastewater, especially in remote settings with no refrigeration capabilities or where these capabilities come with a disproportionately increased cost.

In our previous work, we developed porous superabsorbent polymer (PSAP) beads and applied them as a novel preservation method for biofluid samples (Chen et al., 2022; Chen et al., 2020; Chen et al., 2021). The PSAP beads were rationally designed to have a high water-absorbency (typically about 100–200 g of water per gram of beads) and an average pore diameter of 200–500 nm. With such pore sizes, the PSAP beads can capture small analytical targets (e.g., viruses or their nucleic acids, typically <200 nm in size) while excluding undesired components (e.g., bacteria, typically >500 nm). The captured targets can be recovered by ultrasonication, which breaks the beads, and subsequntly measured. The recovery of >85 % of the bacteriophage MS2 particles and > 95 % of MS2 RNA absorbed in the swelled PSAP beads after 6-week storage at room temperature under controlled laboratory settings (i.e., 0.1 % saline -NaCl- media) were previously demonstrated (Chen et al., 2021). The immobilization of viral capsid on the inner pore surfaces and restrained diffusion rate within the beads by the polymer network resulted in a slow hydrolysis process, which is hypothesized to be the reason for the extended preservation. The abovementioned results demonstrate that PSAP beads can be a feasible option for preserving pathogenic viruses in liquid samples, including raw sewage.

Therefore, in this study, the PSAP beads were applied to absorb real sewage samples and assess the preservation efficiency of SARS-CoV-2 RNA to further determine their applicability for WBE. The novelty of this study is the application of PSAP beads to an important human virus (i.e., SARS-CoV-2) and under realistic settings for WBE (e.g., actual samples from an ongoing surveillance effort), as opposed to spiking-in a known concentration of a bacteriophage previously in the laboratory. The results reported here show that the efficiency of the PSAP beads depends on the exact target used, e.g., different targets show different efficiencies, and is overall high for SARS-CoV-2 without any additional optimizations of the chemical or physical composition of the beads.

2. Materials and methods

2.1. Sample collection of raw sewage

Five hundred milliliters of raw sewage samples were collected from two on-campus sewage maintenance holes (sites I and II, 250 mL per site) adjacent to residential dorms on October 31st, 2022 between 9 and 11 AM as described previously (Cha et al., 2023). Briefly, composite samples were collected in sterile Whirl-Pak bags (part. No. B01195, Whirl-Pak Write On Bags 55 Oz) using HACH autosamplers (AS950, HACH). Total suspended solids (TSS) in the collected composite samples were determined using Standard Method 2540D (Lenore et al., 1998). pH and conductivities were measured using Orion probes (cat. no. 913600 and 013005MD, Thermo Scientific). Site I had a higher TSS concentration of 61.2 mg/L and a higher conductivity of 623.1 μS/cm, which suggested a higher concentration of dissolved salts and possibly other contaminants compared to Site II, which had a TSS of 38.6 mg/L and a conductivity of 292.8 μS/cm. Additionally, the pH value at Site I was 7.71, which was slightly more alkaline than Site II's pH of 7.08. These measurements indicated that Site I's sewage was more concentrated with solids and potentially has a higher load of chemical and biological substances, which affected its basicity and conductivity relative to Site II. Collected sewage samples were stored at 4 °C for 3.5 h before usage.

2.2. Preparation and characterization of PSAP beads

PSAP beads were synthesized as previously reported (Chen et al., 2021). Briefly, 6 wt% sodium acrylate (Sigma-Aldrich), 4 wt% acrylamide (Sigma-Aldrich), 10 wt% polyethylene glycol (PEG, average molecular weight = 6000 g/mol, Sigma-Aldrich), 0.2 wt% Bovine serum albumin (BSA, Sigma-Aldrich), and 0.2 wt% *N,N′*-methylenebisacrylamide (Sigma-Aldrich) were completely dissolved in deionized (DI) water. After being degassed by nitrogen for 5 min to remove the remaining oxygen, 0.3 wt% ammonium persulfate (Sigma-Aldrich) was added into the precursor mixture as the initiator. This precursor mixture was added to a 96-well plate (15 μL per well) and the plate was placed in a bath heater (Thermo Scientific) at 70 °C for 20 min. The formed polymer beads were washed with ethanol thoroughly to remove PEG. Finally, the beads were fully dried at 60 °C.

A scanning electron microscope (SEM, Hitachi SU8010) was applied to observe the morphology and porous structure of the PSAP beads to confirm the successful synthesis. The water absorbency of the PSAP beads in the collected sewage samples at room temperature was measured in triplicate. Briefly, 3 PSAP beads were added to a 20 mL sewage sample, and the weight was measured separately at various time points. The water absorbency was calculated with the following equation,

$$Water\ absorbency = \frac{(m_t - m_0)}{m_0}$$

where m_t is the weight of beads at t min, m_0 is the average weight of three dry beads.

2.3. Performance test of PSAP beads with sewage samples

The viral RNA preservation efficiency of PSAP beads was examined by applying the beads to the collected sewage samples that were spiked with heat-inactivated SARS-CoV-2. We opted to assess preservation up to 14 days to represent the extreme case of wastewater storage, as this period simulates an extended timeframe between wastewater sample collection and analysis in real-world surveillance operations and to assess RNA stability and the efficacy of PSAP beads for RNA preservation over an extended timeframe. Ten microliters of heat-inactivated SARS-CoV-2 (cat. No. VR-1986HK, ATCC) was spiked into 250 mL of sewage

collected from sites I and II. After a gentle swirl and resting on ice for 15 min to homogenize, sewage from each site was aliquoted into thirty 50-mL conical tubes (8 mL per tube). Fifteen tubes were each pre-filled with 70 PSAP beads, and the remaining 15 tubes had no beads (control samples). The aliquoted sewage samples were stored at room temperature (approximately 25 °C) or 4 °C and were sacrificed for measurement on day 0, 7, and 14. To release the viral RNA in the sewage absorbed by PSAP beads, 5 s ultrasonication was applied using a probe sonicator with a frequency of 20 kHz (Qsonica Q125, Newtown, CT). The amplitude of the probe sonicator was set at 75 % and the on/off pulse was programmed at 1 s/1 s. RNA was extracted from 250 μL per sewage sample as previously described (Cha et al., 2023). SARS-CoV-2 RNA targets, N1 and N2, were quantified using a duplex ddRT-PCR assay following previously described methods (Cha et al., 2023; Graham et al., 2020). Fecal strength indicator, pepper mild mottle virus (PMMoV) was also quantified by ddRT-PCR (Graham et al., 2020).

RNA concentrations measured (C_t) were transformed to natural logarithm values after normalizing by the initial concentration (C_0). These ln-transformed values, along with their corresponding time points, facilitated the computation of first-order decay rate constants (k) in units per day via linear regression using the following equation (Bivins et al., 2020; Ahmed et al., 2020):

$$ln\left(\frac{C_t}{C_0}\right) = -kt$$

The adequacy of the linear model was examined using the runs test, and the model's fit was evaluated through r^2 and root mean square error (RMSE). The linear regression and runs test were performed in GraphPad Prism version 10.2.3 for macOS, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

3. Results and discussion

3.1. Basic characterization of the PSAP beads

The dry PSAP beads had a diameter of ~2 mm (Fig. 1A). The SEM image shows the porous structure (Fig. 1B), which was similar to previously reported (Chen et al., 2021). The dendritic structure with interconnected and irregularly shaped pores is due to the increased repulsion between the polymer segments and the high concentration of aqueous PEG solution (Chen et al., 2020). The phase separation process occurs prior to the gel point and porogenic nuclei aggregate, forming a discontinuous polymer network with large pores.

3.2. Absorption of sewage using the PSAP beads

The PSAP beads applied to the sewage from both sites rapidly expanded and reached an absorbency equilibrium in 5 min (Fig. 2). The short equilibrium time allows for the quick sample pretreatment process and promotes the applicability of the bead for virus storage. As shown in Fig. 2, the PSAP beads achieved a water absorbency of 115 ± 3 g/g for Site I and 129 ± 4 g/g for Site II. The slightly lower absorbency for Site I

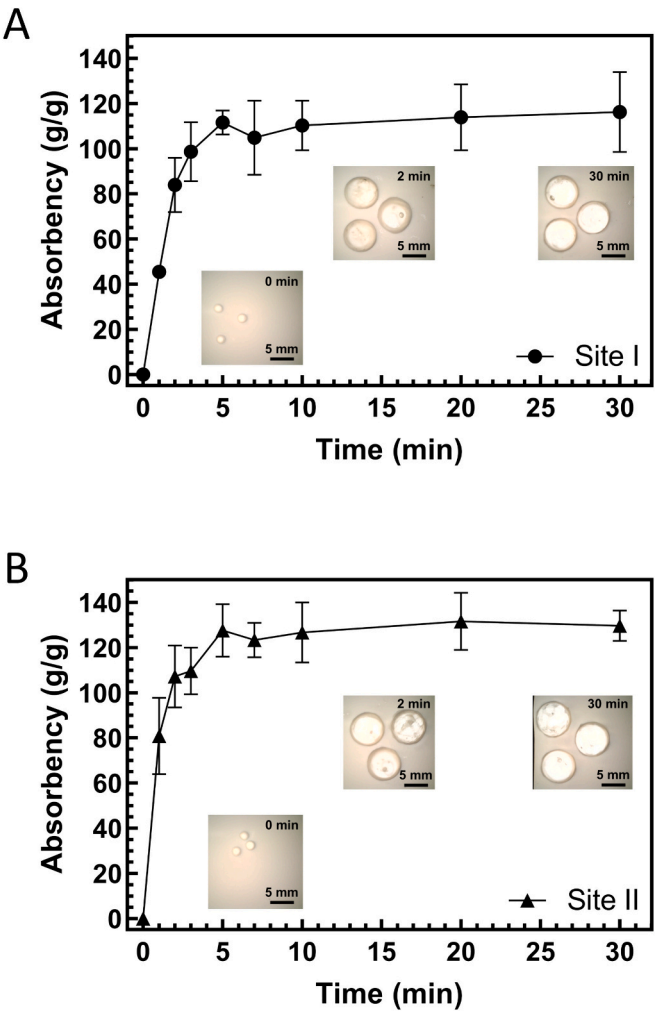

Fig. 2. Absorbency of PSAP beads applied to raw sewage collected from Site I (Panel A) and Site II (Panel B). Triplicate experiments using three PSAP beads were conducted for each site. The inset images show the change in the diameters of PSAP beads along the swelling process.

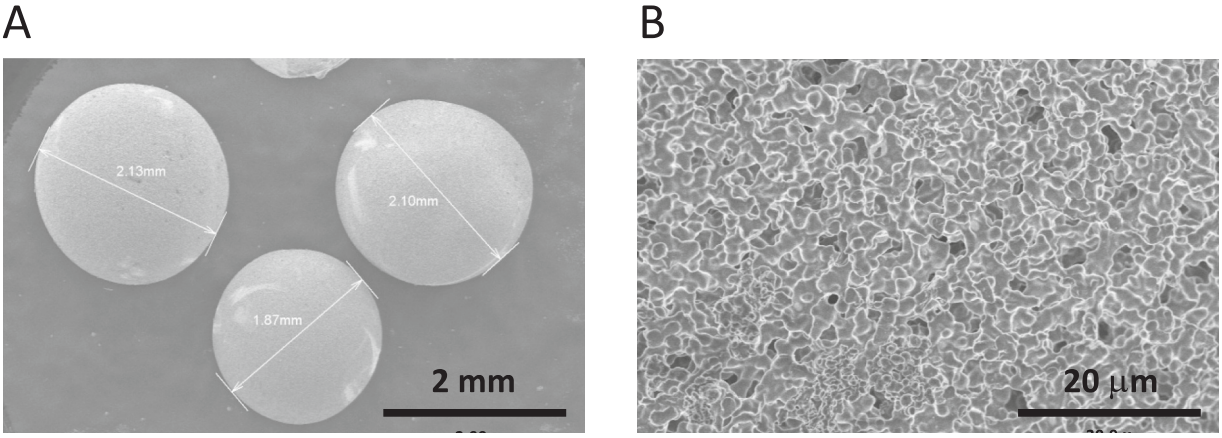

Fig. 1. SEM images of dry PSAP at low (A) and high (B) magnifications.

can be explained by the higher suspended solids and higher ion concentration of the wastewater. Suspended solids might have blocked the water channels in the beads, and the higher ion concentration in bulk solution could have resulted in an increased equilibrium osmotic pressure and a decreased water absorbency.

3.3. Recovery of viral RNA from PSAP beads

Once the PSAP beads were applied to the sewage samples, the liquid and suspended materials with the size smaller than the surface pores of the PSAP beads - including the viral RNA - were absorbed into the inner volume. The recovery efficiency of the PSAP beads was determined by dividing the viral RNA concentrations in day 0 sewage retrieved from the PSAP beads by the viral RNA concentrations in day 0 control samples (Fig. 3). For this, previously validated ddRT-PCR assays (Cha et al., 2023; Graham et al., 2020) were used to quantify SARS-CoV-2 abundance (copy number) based on the N1 and N2 gene targets, and PMMoV abundance. Note that our approach does not account for the recovery of the spiked-in SARS-CoV-2 for counting (e.g., what fraction of the total spiked in copies was successfully processed by our wet-lab methodology and counted). However, if the recovery is similar between the untreated and treated by PSAP beads samples, as it is reasonable to expect, there will be no difference in the ratios reported here.

The mean recovery efficiencies of the three viral RNA targets from PSAP beads ranged from min 88.7 ± 6.3 % (N2 in site I sewage) to max 96.7 ± 14.0 % (N2 in site II sewage). Considering the pore size of the beads, not only were bacteria and grazers excluded from the material absorbed by the beads, but also any viral material that might have adhered to particulates larger than the pore size of the beads. The slight loss of viral RNA compared to the recovery efficiency for the model virus in synthetic medium (Chen et al., 2022; Chen et al., 2021) is likely attributable to the fraction of virions and viral RNA adhered to those large solid particulates.

3.4. Preservation of SARS-CoV-2 viral RNA

Viral RNA concentrations measured from the control or PSAP-treated sewage samples before and after storage at 4 °C or room temperature are shown in Fig. 4. The first-order decay curves and rate constants are provided in the supplementary material (Fig. S2 and Table S2). The preservation effect by PSAP beads was not significantly different compared to the control at 4 °C as both control and PSAP beads had comparable N1 retention at ~80 % after 14 days (Fig. 4A) and comparable decay rate constants of 0.37–0.49 day^{-1}. However, PSAP beads had a clear positive effect on the preservation of SARS-CoV-2 N1 in sewage under room temperature. After two-week storage at room temperature, 33 ± 10 % reduction of N1 was observed in sewage retrieved from PSAP beads compared to the initial state. In contrast, 66 ± 10 % reduction was observed in the control. The higher decay rate constants in the control (1.6–2.2 day^{-1}) compared to the PSAP-treated sewage (0.56–0.83 day^{-1}) further indicate the faster decay of RNA without PSAP treatment at room temperature (Table S2). The decay of SARS-CoV-2 RNA in the control samples is also comparable to the previously reported T90 value of 3.3 days when high titer SARS-CoV-2 RNA was incubated in untreated wastewater at 25 °C (Bivins et al., 2020). The N2 assay showed overall comparable trends in terms of preservation as the N1 assay (Fig. 4B and Table S2). The observed enhanced preservation of SARS-CoV-2 RNA by PSAP treatment can be explained as a result of microfiltration of unwanted bacterial and protozoan cells in sewage by surface pore structures of PSAP beads during the rapid swelling of sewage within 5 min, and the effective immobilization and stabilization of viral particles and nucleic acids by virus-polymer interactions in the inner surface (Chen et al., 2022).

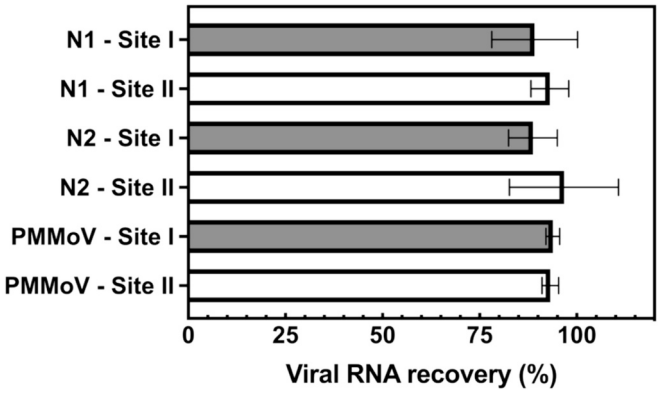

Fig. 3. Recovery of viral RNA from PSAP beads. The mean and standard deviation of recovery efficiency of viral RNA from PSAP beads (x-axes) from triplicate sewage samples collected from Site I or Site II are shown. The recovery efficiency represents the ratio of the viral RNA concentration in PSAP-treated Day 0 sample over the Control Day 0 sample.

In the case of PMMoV, its decay in PSAP beads or controls was consistently slower compared to SARS-CoV-2 RNA in both sites regardless of the storage temperature (Fig. 4C and Table S2). The greater preservation of PMMoV compared to SARS-CoV-2 in raw sewage both with and without PSAP beads treatment can be attributed to their structural differences. Consistent with this hypothesis, previous studies have shown that the envelope structure of SARS-CoV-2 makes it more sensitive to environmental stresses like temperature and chemical exposure, leading to faster decay rates in sewage. In contrast, PMMoV lacks an envelope, which contributes to its increased stability and resistance under similar conditions (Li et al., 2023; Ye et al., 2016).

4. Conclusions

In this work, we demonstrated the effectiveness of PSAP treatment for preserving viral RNA, especially of SARS-CoV-2, in raw sewage matrix. The preservation effect was not significant in sewage stored at 4 °C compared to the control samples. However, the decay of SARS-CoV-2 RNA concentrations was significantly lower in sewage retrieved from PSAP beads compared to control when stored at room temperature. Therefore, our results demonstrate the applicability of PSAP beads to WBE projects for preservation of SARS-CoV-2 RNA in raw sewage, especially in remote settings with no refrigeration capabilities. While our study primarily investigates the preservation technique for SARS-CoV-2, it is important to note that our findings may have broader applications. This approach could potentially be adapted for tracking a variety of viral pathogens in wastewater systems, enhancing our capability to monitor public health. Future work could focus on elucidating the exact mechanism of preservation by PSAP beads (e.g., absorption mechanism of RNA or viral capsids) toward further modifying the surface of the bead for specific microbial/viral targets and/or higher efficiency.

CRediT authorship contribution statement

Gyuhyon Cha: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yixuan Huang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Katherine E. Graham:** Methodology, Investigation, Data curation, Conceptualization. **Anjin Luo:** Methodology, Investigation. **Wensi Chen:** Methodology, Investigation. **Janet K. Hatt:** Validation, Resources. **Konstantinos T. Konstantinidis:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Xing Xie:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

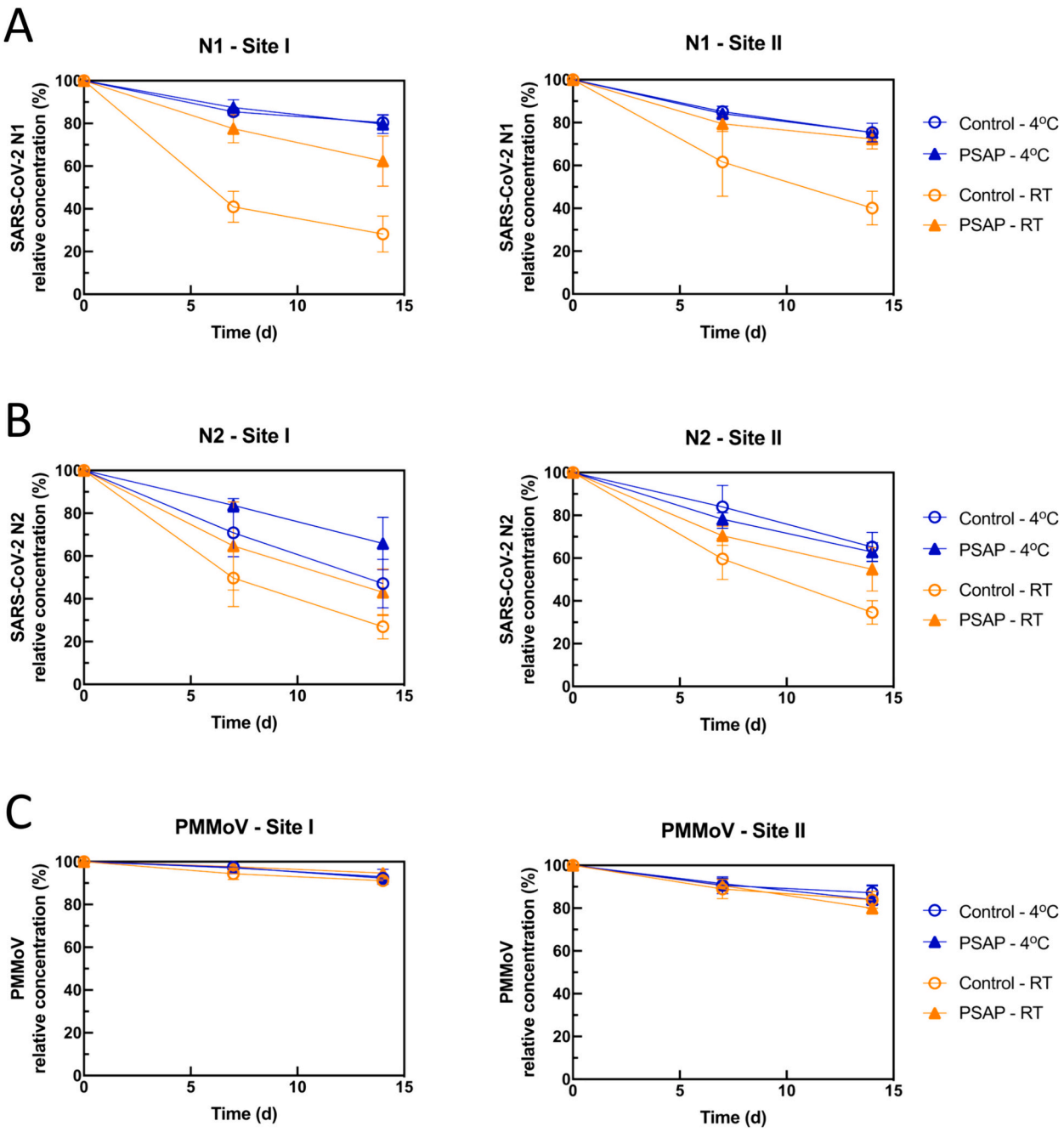

Fig. 4. Preservation of viral RNA by PSAP beads. Sewage samples collected from two on-campus sewage maintenance holes (Site I and II) were stored at 4 °C or room temperature (RT, approximately 25 °C) for 2 weeks; the samples were either processed with PSAP beads or not (control). The mean and standard deviation of relative concentrations (y-axes) of SARS-CoV-2 N1 (Panel A), SARS-CoV-2 N2 (Panel B), and PMMoV (Panel C) based on ddRT-PCR assays from triplicate sewage samples collected from Site I or Site II are plotted against storage duration (x-axes). The relative concentration of the viral RNA was determined by comparing the concentration of the viral RNA in the composite samples stored for 7 or 14 days, either released from the PSAP beads or free-floating (control), to the concentration in the original composite sample at $t = 0$.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.173468.

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