RESEARCH ARTICLE

Seasonal occurrence and fate of nanoparticles in two biological wastewater treatment plants in Southern California

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Abstract

Nano-sized particles in wastewater are generally considered colloids, but their production and size distribution are not well understood. Organic nano-sized particles are more abundant than engineered nanomaterials in wastewater, where they may cause membrane fouling, harbor pathogens, and transport contaminants to the environment. To our knowledge, this study is the first to examine the seasonal behavior, removal, and the quantity and size of suspended particles (both unfiltered and filtered through a 450 nm filter) at multiple points within different processes along two water resource recovery facilities (WRRFs, formerly wastewater treatment plants). In Southern California where wastewater is often reused or reclaimed, a better understanding of nano-sized particles generation and removal may help reduce cost. We found that both types of the biological secondary treatments investigated (conventional activated sludge process and trickling filter) were more efficient in removing suspended particles larger than 450 nm than they were smaller ones. However, the results show that current treatment processes are not designed to remove nano-sized particles efficiently. We also investigated the factors that correlate with their occurrence and found that there was a significant and direct correlation between influent dissolved chemical oxygen demand (COD) and the abundance of suspended particles both larger and smaller than 450 nm, suggesting that the suspended particles increased with dissolved COD in the WRRFs and thus were biogenically generated during the wastewater treatment. Although no conclusive seasonal correlations were found, dissolved COD management may control nano-sized particle production.

Practitioner Points

- Conventional secondary treatments (activated sludge and trickling filter) could efficiently remove particles but not as efficiently for nano-sized particles (40.1–52.7% removal).

Josh Smeraldi and Linda Y. Tseng contributed equally as first authors.

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- At one facility, particles of all sizes were found to correlate with dissolved carbon and EPS, meaning they were biogenic.
- Monitoring dissolved carbon or EPS precursors may help control membrane fouling post-secondary treatment, and this warrants more studies.

KEYWORDS

activated sludge process, extracellular polymeric substances, nanoparticles, wastewater treatment, water resource recovery facility

INTRODUCTION

Naturally occurring nanoparticles (NONPs) are found in the environment in concentrations orders of magnitude higher relative to engineered nanomaterials (ENMs) (Bernhardt et al., 2010; Montaño et al., 2014; Nowack & Bucheli, 2007; Sun et al., 2014; Wagner et al., 2014). However, more research studies have focused on the fate and transport of ENMs rather than on NONPs because of ENMs′ novel properties and potential impact on the natural environment (Bernhardt et al., 2010; Cappadona, 2020; Cervantes-Avilés & Keller, 2021; Chen, 2022; Floris, 2017; Huang et al., 2021; Khan et al., 2022; Lange et al., 2020; Nowack & Bucheli, 2007; Roth et al., 2015). Additionally, NONPs have been generally studied as colloids within wastewater treatment processes where the particles may be composed of a large variety of materials (Dean, 1969; Dean et al., 1967; Kowalkowski, 2010; Kretzschmar et al., 1994; Kretzschmar et al., 1997; Kretzschmar & Sticher, 1997, 1998; Lead & Wilkinson, 2006; Prestel et al., 2005; Rickert & Hunter, 1971; Song et al., 2010) and sizes (Aiken et al., 2011; Cervantes-Avilés & Keller, 2021; Lead & Wilkinson, 2006; Mohlman & Pearse, 1922; Prestel et al., 2005); this may be due to the fact that the definitions for colloids and nanomaterials overlap, and there is no established distinction between the two terms (Bar-Zeev et al., 2009; Grossart et al., 1997; Passow, 2002; Song et al., 2010; Wilkinson et al., 1997). Colloids are defined by some as small particles that are suspended and do not stabilize well, with sizes ranging from 1 nm to 1 μm (Lead & Wilkinson, 2006; Mohlman & Pearse, 1922). In contrast, nanomaterial is broadly defined by the United States Environmental Protection Agency and the European Commission (EC) as material between 1 and 100 nm in at least one dimension (EC, 2011; USEPA, 2021), with recognition that there are NONPs. As a result, "nanoparticles" were most likely part of many "colloids" studies, and these terms were used interchangeably in past research. For example, biogenic organic nanoscale materials from a blend of groundwater and final effluent of an activated sludge wastewater treatment were found to resemble polysaccharide-like colloids in freshwater (Song et al., 2010). Since the definitions of colloids and nanoparticles overlap, in this study, we used 0.45-μm filter and the Zetasizer Nano ZS to aid our understanding of small particle size and count distribution in the wastewater, knowing that this filter size cut-off would cover a range of different particles such as the dialysis membrane 35,000 Da cut-off for biogenic organic nanoscale materials and dissolved organic nitrogen, the 0.9-μm cut-off for dissolved organic matter (Leenheer et al., 2007; Mash et al., 2004; Song et al., 2010), and 0.45-μm cut-off for dissolved organic matter and protein in the wastewater (Mohapatra et al., 2021). We refer to particles of size range 1 to 100 nm as nanoparticles.

Nanoparticles and colloids, depending on their physical, chemical, and biological properties, can have adverse effects on wastewater treatment and the quality of treated effluent (Dean, 1969; Dean et al., 1967; Kowalkowski, 2010; Lead & Wilkinson, 2006). It has been well studied that colloids can cause membrane fouling in wastewater treatment processes (Amy, 2008; Cogan et al., 2022; Jarusutthirak et al., 2002; Lee et al., 2005; Lesjean et al., 2005; Meng et al., 2006; Meng et al., 2009; Rosenberger et al., 2006; Safarik & Phipps, 2005). In particular, many of the nano-sized suspended particles in wastewater are organic (Kowalkowski, 2010; Lead & Wilkinson, 2006; Song et al., 2010) (e.g., protein and polysaccharide) and are thought to be part of soluble microbial products and extracellular polymeric substances (EPSs) (Bar-Zeev et al., 2009; Mohapatra et al., 2021; Passow, 2002; Wang et al., 2009), both of which have been found to cause reversible as well as permanent fouling on filtration membranes even after backwash cleaning (Amy, 2008; Cogan et al., 2022; Jarusutthirak et al., 2002; Meng et al., 2009; Rosenberger et al., 2006). This type of fouling increases the cost of treatment, either in the form of increased energy demand over time (Akhondi et al., 2014; Chua et al., 2002; Gander et al., 2000; Krzeminski et al., 2012) or in the form of the material cost of cleaning or replacing the filtering membrane (Guo et al., 2012; Meng et al., 2009). In addition,

colloids can cause light scattering resulting in inadequate disinfection by UV and also impair the esthetic quality of the treated effluent (Christensen & Linden, 2003; Gilboa & Friedler, 2008; Hassen et al., 2000; Passantino et al., 2004). The scattering of UV also means colloids may also hinder the photodegradation of certain contaminants in the wastewater (Mohapatra et al., 2023). During chlorination specifically, colloidal particles may harbor and shield pathogens (Winward et al., 2008), and if they are organic colloidal particles, they may also increase the chlorine demand of the treated effluent (de Beer et al., 1994; Falsanisi et al., 2008; Gautheir et al., 1999) and even form *N*-nitrosodimethylamine (Dotson et al., 2009; Mitch & Sedlak, 2004). Furthermore, smaller particles have high specific surface area, which provides a higher number of potential adsorption sites for many contaminants (Chen et al., 2006; Khan et al., 2021; Kowalkowski, 2010; Rudolfs & Zuber, 1953; Theng & Yuan, 2008; Zhang et al., 2007); if the colloids are not removed during the treatment process, they could enhance the transport of contaminants to the environment (Bolong et al., 2009; Brown et al., 2007; Carballa et al., 2005; Darwano et al., 2014; de Souza et al., 2017; Maskaoui & Zou, 2010; Okuda et al., 2009; Paulson et al., 1984; Petrovic et al., 2004; Rimkus, 1999; Senta et al., 2013; Stasinakis et al., 2013; Sumner et al., 2010; Thomas & Forster, 2005; Wijayaraine & Means, 1984; Winkler et al., 1998; Yang et al., 2011; Zgheib et al., 2011). Making matters even more complicated, colloids in wastewater can contain anthropogenically generated antibiotic resistance genes and may therefore potentially contaminate drinking water sources or transfer these genes to pathogenic bacteria if the colloids are not removed properly (Riquelme Breazeal, 2011). Some have also found organic colloids, particularly those from the wastewater effluent, contributing a substantial nitrogen load to sensitive environments (Bradley et al., 2010; Bronk et al., 1994; Bronk et al., 2010; Leenheer et al., 2007; Pehlivanoglu & Sedlak, 2004). However, few studies have investigated the long-term production and treatment efficiencies of nanoparticles in wastewater within different treatment processes.

With more water reuse and reclamation efforts from treated wastewater effluent underway due to drought, understanding particles and nanoparticles production is important to reduce potential adverse effects on wastewater effluent quality and the impact on wastewater treatment (e.g., disinfection and membrane fouling). The dearth of literature on the production and behavior of particles of different sizes in water resource recovery facilities (WRRFs, formerly wastewater treatment plants) means our knowledge to predict the nanoparticle production in wastewater treatment is still incomplete (Nowack & Bucheli, 2007; Wagner et al., 2014). This study aims to understand the seasonal prevalence and size of particles along the wastewater treatment process and comparatively analyze them with the water quality parameters and activated sludge EPS. This study also aims to understand the removal of particles in each treatment process. Although there are studies on monthly variation of inorganic nanoparticles in wastewater (Chen, 2022), albeit few, to our best knowledge, this study is the first report on the seasonality of general nanoparticles in fine detail at multiple locations within each treatment processes in WRRFs and nanoparticles' correlation with EPS production.

MATERIAL AND METHODS

Sampling sites

We investigated the fate and transport of particles at two Southern California WRRFs. Plant 1 had an equalization basin operated with the modified Ludzack–Ettinger (MLE) configuration, with an internal recirculation from the end of the activated sludge process (ASP) to the beginning of the ASP (Figure 1). Full denitrification was achieved through methanol addition at this plant, which treated approximately $6 \cdot 10^4$ m^3 day^{-1} (~16 MGD) and had a yearly average mean cell retention time (MCRT) of 8.5 days as well as an average hydraulic retention time (HRT) of 6 h. Following tertiary treatment using a sand filtration and chlorination, most of the flow was reclaimed. There were several points during the treatment process where chemicals were added in Plant 1: The primary clarifier had a ferric dosage of 26 mg L^{-1}, and when the flow was more than $5.7 \cdot 10^4$ m^3 day^{-1} (~12.5 MGD), an additional 0.2 mg L^{-1} of polymer was added. Here, 8 mg L^{-1} of methanol was added at the beginning of ASP into the anoxic denitrification zone, the return activated sludge was fed magnesium chloride (MgCl$_2$) at 20 mg L^{-1} to balance the pH, and alum was added to the secondary effluent after the secondary clarifier and before tertiary granular filtration.

Plant 2 did not have an equalization basin, but it had a Ludzack–Ettinger (LE) layout (identical to Plant 1 but without internal recirculation) and performed partial denitrification and nitrification. No supplemental carbon was added in this process. The plant capacity was approximately $2.3 \cdot 10^4$ m^3 day^{-1} (~6.1 MGD) and had a yearly average MCRT of 3.3 days as well as an average HRT of 4.4 h (Figure 1). Approximately $1.9 \cdot 10^4$ m^3 day^{-1} (~5 MGD) from the primary effluent went to the ASP and the rest went to trickling filters (TFs). The stream that went through the TF would go through the TF secondary
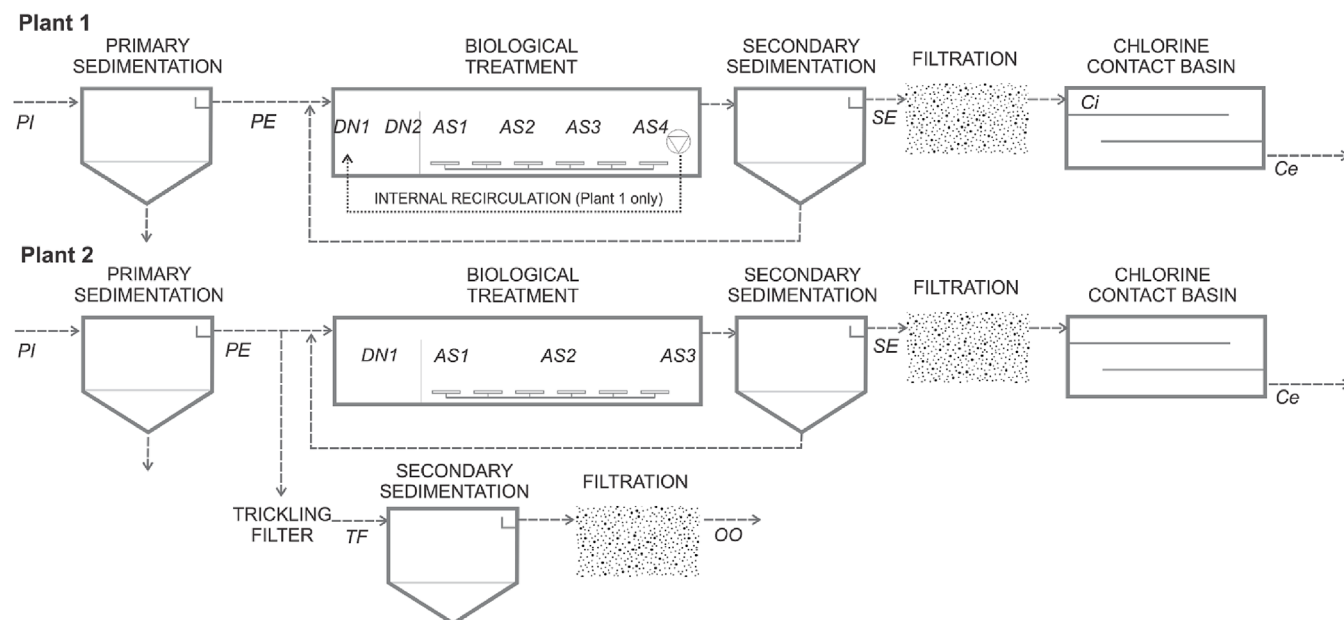
FIGURE 1 Schematic of the process configuration of Plants 1 and 2. There were 11 sampling locations at Plant 1: primary influent (PI), primary effluent (PE), preanoxic denitrification (DN1 and DN2), aerated activated sludge (AS1, AS2, AS3, and AS4), secondary effluent (SE), chlorine contact basin influent (Ci), and chlorine contact basin effluent (Ce). There were 10 sampling locations at Plant 2: PI, PE, DN1, AS1, AS2, AS3, SE, Ce, trickling filter effluent (TF), and effluent to the ocean outfall (OO).

clarifier and would be discharged to the ocean, and the stream from the ASP was reclaimed after being filtered through a mesh cloth. There was alum addition in the secondary effluent after the secondary clarifier and before filtration.

Table S1 shows the typical primary effluent quality for both sites.

Sampling and laboratory analyses

One-liter grab samples were taken with a sampling stick that was rinsed three times with the sample water before the sample was taken. Samples were transported in Whirl-Pak bags (Nasco, Wisconsin) and taken monthly for a period of 1 year and were processed the same day. Samples were collected during the same day of the week at the same time of day to minimize inconsistencies. If there was a precipitation event 72 h prior to sampling, sampling was postponed to at least 72 h post-precipitation (results from the second February are missing due to continuous rain). Sampling points within both plants are indicated in Figure 1. The temperature and oxidation–reduction potential of samples were measured immediately after samples were taken.

For the particle analysis, each sample was allowed to settle for 1 h, and then, the supernatant containing unsettled particles was collected ("larger particles in unfiltered sample"). Part of the same supernatant was filtered

through a Whatman 934-AH 0.45-µm glass fiber filter ("nano-sized particles in filtered sample") to remove larger particles that might interfere with the analysis of smaller particles. The mean particle size (Z_{avg}, nm; intensity weighted mean hydrodynamic size) and count (count rate, CR, kcps) of both filtered and unfiltered supernatant were analyzed using Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) (Smeraldi et al., 2012). The Malvern Zetasizer Nano ZS utilizes dynamic light scattering technology to monitor the Brownian motion of nanoscale particles and produces a nanoscale size distribution curve of particles suspended in a sample. It was previously determined that a surrogate particle count of the sample can also be determined using the photon count output from the instrument (Smeraldi et al., 2012). The filtered and unfiltered supernatants were also analyzed for chemical oxygen demand (COD) (American Public Health Association, 2005). We did not obtain COD data of the supernatant in the first 2 months (February and March) for the unfiltered samples and also did not during October for some filtered and unfiltered samples.

For the EPS analysis, EPSs of the activated sludge were extracted according to Li and Yang (2007) and Tseng et al. (2015) and separated into loosely bound (LB EPS) and tightly bound (TB EPS) fractions. Briefly, 50 ml of well-mixed sludge samples was transferred to 50-ml polypropylene centrifuge tubes and underwent 5 min of centrifugation at $4 \cdot 10^3$ g. The supernatant was discarded and replaced with the same volume using a
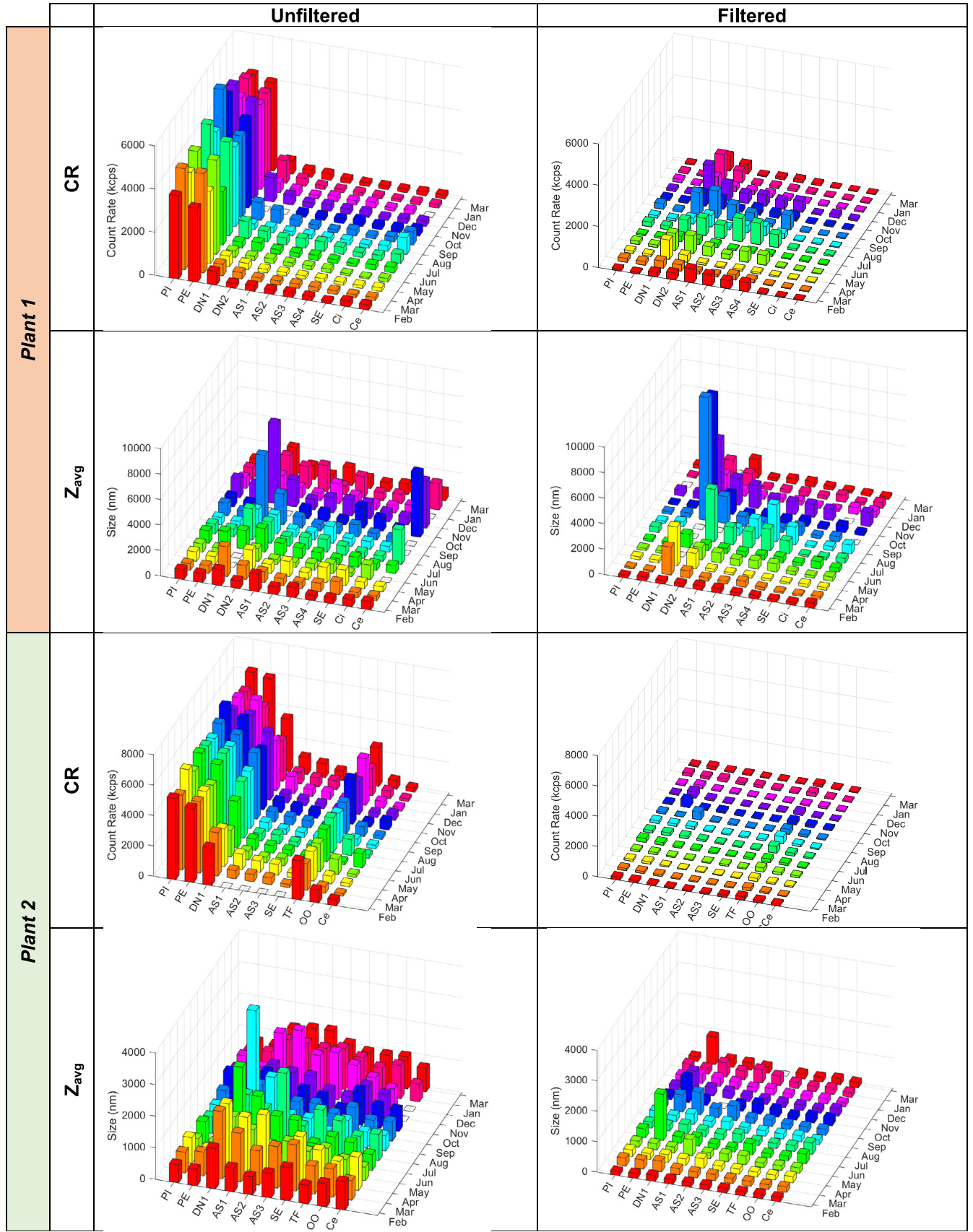
FIGURE 2 Legend on next page.

FIGURE 2 Particle count (CR) and average size (Zavg) for unfiltered and filtered (by a 450-nm filter) sample supernatants over the course of 13 months along Plants 1 and 2 at the following points: primary influent (PI), primary effluent (PE), preanoxic denitrification (DN1 and DN2), aerated activated sludge (AS1, AS2, AS3, and AS4), secondary effluent (SE), chlorine contact basin influent (Ci), and chlorine contact basin effluent (Ce).

$50°C$ solution of 0.05% sodium chloride (NaCl), which was prepared with $18.2\,M\Omega\,cm^{-1}$ water. After resuspending the pellet with a vortex mixer for 1 min, the samples underwent centrifugation for 10 min at $4 \cdot 10^3 g$. The supernatant subsequently obtained here was considered to be loosely bound EPS (LB EPS). The supernatant was replaced with fresh $60°C$ 0.05% NaCl solution and heated in a water bath at $60°C$ for 30 min. After centrifugation at $4 \cdot 10^3 g$ for 15 min, the supernatant obtained was considered to be the tightly bound EPS (TB EPS). Each EPS fraction was quantified by its protein and polysaccharide content using the procedures from the Lowry et al. (1951) assay and DuBois et al. (1956), respectively. Since this study sought to examine the occurrence of nano-sized particles with the production (not the morphology) of EPS in the wastewater, we only sought to analyze major EPS components found in a previous study, polysaccharides, and protein (Tseng et al., 2015).

To further investigate the temperature and the seasonality effect on the Z_{avg} and CR, the study period was separated into cooler months (December to April) and warmer months (May to November).

Statistical analysis

Our results were analyzed with Pearson's correlation for significance ($p < 0.05$) between two paired parameters and further analyzed with principal component analysis (PCA) biplots to understand multi-parameter correlations (MATLAB R2018a). To perform PCA on our complete data set required our data to be free of missing data. However, there were some missing data points in the first month of our study because some sampling points were added during the second month to have a finer resolution of the suspended particle behavior, and there were also some reporting irregularities from the instruments (i.e., values shown as out of range; missing data shown as colorless bar spaces in Figure 2). Therefore, data augmentation was performed to analyze the full data set. The missing data values were augmented as follows: We first separated cooler and warmer months because the plant operating conditions were different in those months, and then, we interpolated the missing data found in the warmer months by averaging the data in the previous month and the following month. The same procedure was applied to the cooler months. Since the data missing

in the beginning of the sampling campaign could not be augmented with the average from the previous month, we augmented such missing data with the average of the existing data points. The PCA coefficient results from the original and the augmented data were compared using the chi-squared statistical test to check whether they were significantly different ($p < 0.05$).

RESULTS AND DISCUSSION

Monthly trend

The particle count and average size of particles in the monthly wastewater samples are plotted in Figure 2 for both Plants 1 and 2.

Particle CR and removal efficiency in each treatment process

In Plant 1, we found that the CR in the unfiltered samples was generally much higher in the primary treatment samples relative to samples from the secondary and tertiary treatment (Figure 2; Table S2). The differences observed were possibly due to the ferric and polymer added during the primary treatment. However, after filtering through the 0.45-μm pore size filter, the average CR in the secondary treatment samples was much higher than in the primary treatment samples. This indicated that in Plant 1, most of the nano-sized particles were produced by the ASP and were not already present in the influent wastewater to the plant. When reviewing monthly variations in the CR for both filtered and unfiltered samples, there were slightly more particles observed in the warmer months (CR $1,027 \pm 1,663$ and $1,163 \pm 1,113$, respectively) than in the cooler months (CR 442 ± 524 and 942 ± 496, respectively), suggesting a possible temperature or seasonal dependence for particle production. Although Chen (2022) and Mohapatra et al. (2021) both studied monthly and seasonal variation of nanoparticles, Chen targeted inorganic nanoparticles such as titanium nanoparticles, whereas Mohapatra et al. targeted only DOM. Since this study, to our knowledge, is the first study to report potential temperature dependence of all nanoparticles in wastewater, there are no other studies we could use to corroborate our finding. The statistical significance of potential

temperature and seasonal effect on CR will be discussed in the Statistical Analysis section.

As a whole, Plant 1 was more efficient at treating larger particles than removing nano-sized particles, removing 93% of larger particles and 59% of nano-sized particles (Table S3). The overall secondary treatment of Plant 1 (ASP and secondary clarifier) had a higher treatment efficiency for larger particles (95.9% relative removal) than for nano-sized particles (40.1% relative removal), but secondary treatment was the most effective process in removing both the larger and nano-sized particles out of all the treatment processes at Plant 1, suggesting that sedimentation was integral in particles removal in wastewater treatment. The secondary clarifier by itself was more efficient in removing nano-sized particles (64.5% relative removal) than larger particles (26.9% relative removal). In addition, while tertiary chlorination process removed some nano-sized particles, the treatment had an increased abundance of larger particles. The results suggest that larger particles may be produced within the tertiary treatment post-secondary sedimentation and post-secondary treatment filtration.

Removal efficiency of both larger and nano-sized particles was higher during warmer months in primary treatment and secondary clarifier (Table S3). The removal of nanosized particles was more efficient in the tertiary treatment during warmer months. However, as mentioned before, the abundance of nano-sized particles increased in ASP in warmer months (Table S2). This suggests that temperature and seasonality might have influenced the particle production and removal differently during individual unit processes in Plant 1.

In Plant 2, similar to Plant 1, the CR of the primary treatment unfiltered samples was generally higher than that of the secondary and tertiary treatment samples (Figure 2; Table S2). Also similar to Plant 1, Plant 2 was more efficient overall in treating larger particles (94.1% removal) than removing nano-sized particles (43.9% removal) (Table S3). The overall secondary treatment via ASP and via TF were both more efficient in removing larger particles (95.4% and 88.9% relative removal, respectively) than nano-sized ones (52.7% and 28.3% relative removal, respectively), but the overall secondary treatment via ASP removed more particles than did overall secondary treatment via TF. The high particle removal efficiency in the secondary treatment, regardless of ASP or TF, again suggests that sedimentation is important in particle removal. The results from the secondary treatment are very similar to those found in a report on three treatment plants in the same region of Southern California surveyed for their nanoparticles removal, with their ASP having 63.9% and 85.5% removal and TF 74.5% removal (Rosso & Rajagopalan, 2011). This means the removal efficiencies may vary within a range and small differences between removal efficiencies may not be significant. Comparing to silver nanoparticles in wastewater from a recent study, silver nanoparticles in the primary effluent were shown to be mostly removed after ASP and secondary clarifier (91% compared to nano-sized particle removal from Plant 1 by overall secondary treatment 40.1%, Plant 2 52.7% in this study) (Cervantes-Avilés & Keller, 2021). This suggests that secondary clarifier and sedimentation are better at removing metal ENMs than the general nano-sized particles present in wastewater treatment. In fact, since many metal ENMs can aggregate and agglomerate, most of the metal ENMs have very high removal efficiencies in the wastewater treatment; thus, the nano-sized particles found in the wastewater treatment were inherently different from metal ENMs (Cervantes-Avilés & Keller, 2021; Chen, 2022; Ganesh et al., 2010; Huang et al., 2021). The tertiary chlorination removed additional nano-sized particles (11.1% relative removal) but increased the abundance of larger particles (46.2% relative increase), which also suggests that larger particles may be formed during chlorination as seen at Plant 1. The simultaneous decrease of nano-sized particles and the formation of larger particles could mean that coagulation was occurring during chlorination.

The similar abundance of nano-sized particles during the primary treatment in Plant 2 and in the beginning of the ASP was not seen in Plant 1 (Figure 2; Table S2), perhaps because there was no addition of chemicals and no equalization basin in Plant 2. Although there was no chemical added, the overall ASP treatment in Plant 2 was more efficient in removing nano-sized particles than in Plant 1 (Table S3).

Warmer temperatures increased the abundance of nano-sized particles in ASP secondary clarifier in Plant 2 (Table S3), suggesting warmer temperatures may have negatively impacted the removal efficiency of nano-sized particles in ASP secondary clarifier. However, warmer temperature seemed to increase the removal efficiency of nano-sized particles from the TF secondary clarifier. The analysis of potential temperature and seasonal effect on CR in both Plants will be discussed in the Statistical Analysis section.

Average particle size (Z_{avg})

The Z_{avg} in Plant 1 was generally smaller in the filtered samples than in the unfiltered samples (Figure 2; Table S4). Within the treatment train, the mean Z_{avg} was the largest in the ASP for both filtered and unfiltered samples. In the entire secondary treatment, the unfiltered samples had a majority of the particles with a size below 100 nm (particles with size below 100 nm in unfiltered

samples 15.9% $\pm$ 9.0%, 55.1% $\pm$ 33.3%, and 38.8% $\pm$ 34.9% for primary, secondary, and tertiary treatment, respectively). For the filtered samples, the percentage of particles with size below 100 nm had a different trend compared to unfiltered samples (particles with size below 100 nm in filtered samples 40.1% $\pm$ 32.5%, 36.1% $\pm$ 28.8, and 58.4% $\pm$ 24.9% for primary, secondary, and tertiary treatment, respectively). Although the filtered and unfiltered samples showed different trends, the percentage of particles with size below 100 nm increased at the end of the tertiary treatment compared to the beginning of the primary treatment. However, when comparing the Z_{avg} of the particles in the beginning and at the end, the mean Z_{avg} was larger at the end of the treatment than in the beginning (Figure 2; Table S4). The increased percentage of particles with size below 100 nm at the end of Ce was not due to larger particles breaking up in the secondary treatment, mass balance-wise, because a larger volume of larger particles was removed than the volume of smaller particles was produced (Figure S1). Instead, it was likely due to different processes that started in the secondary treatment because after a huge increase of Z_{avg} during the secondary treatment, there was a consistent decrease of Z_{avg} along the treatment train, even though the Z_{avg} in the tertiary treatment was larger than in the beginning (Figure 2; Table S4), perhaps due to coagulation. The increase of particles less than 100 nm may present a challenge for filtration post-secondary treatment, because sizes less than 450 nm was found to be more important for causing membrane fouling (Howe & Clark, 2002). In terms of the temperature effect, as alluded to by the CR results in Plant 1, the average particle size seemed to be larger during the warmer months than in the cooler months.

In Plant 2, the majority of Z_{avg} was smaller in the filtered samples than in the unfiltered samples (Figure 2; Table S4). For unfiltered samples, the largest Z_{avg} of every month was found in the ASP; for filtered samples, about half of the largest Z_{avg} of the month occurred in the primary treatment processes, while the other half occurred in the ASP. For filtered samples, the percentage of particles with size below 100 nm increased more compared to unfiltered samples (volume percentage of particles with size below 100 nm in unfiltered samples in primary treatment 10.6% $\pm$ 18.0%, ASP 36.5% $\pm$ 36.7%, TF 22.3% $\pm$ 27.8%, tertiary treatment 53.2% $\pm$ 32.1%; in filtered samples in primary treatment 30.4% $\pm$ 28.2%, ASP 61.1% $\pm$ 22.6%; TF 47.0% $\pm$ 18.7%, tertiary treatment 61.5% $\pm$ 19.9%). For both filtered and unfiltered samples, the percentage of particles with size below 100 nm increased at the end of the tertiary treatment, and this increase is similar the results from Plant 1. Similar to Plant 1, warmer months seemed to produce a slightly larger

Z_{avg} than cooler months. This could mean that temperature may have an influence on the size of the particles. Unlike Plant 1, however, Z_{avg} at the end of the treatment was similar to the beginning of the treatment.

With particles below 100 nm still present in both plants at the end of the tertiary treatment, and the overall removal of nano-sized particles via ASP being 59.0% at Plant 1 and 43.9% at Plant 2 (1.3% via TF; Table S3), our results suggest that current treatment processes are not designed to efficiently remove nano-sized particles, though the overall treatment seems to be quite efficient in removing larger particles (93.0–94.1% removal via ASP, 89.9% via TF; Table S3). Interestingly, the removal efficiencies of larger particles seem to also coincide with the removal efficiency of microplastic particles and fibers from wastewater (1–5 mm, with 100–5 mm being the majority; 84–97% removal) (Magni et al., 2019). Therefore, any improvements to the current treatment processes to remove nano-sized particles should also be cognizant of the removal of other particulate matter of concern. The dramatic size change behavior was not observed in metal ENMs, where most of the metal ENMs had very similar sizes from the beginning of the wastewater treatment to the end (Cervantes-Avilés & Keller, 2021; Chen, 2022; Huang et al., 2021). This again suggest that most of the nano-sized particles in the wastewater treatment stream respond differently than metal ENMs in wastewater treatment.

In a few cases, the Z_{avg} of the filtered samples was larger than unfiltered samples in Plant 1 (Figure 2; Table S4), and some Z_{avg} from filtered samples was even larger than the filter pore size (0.45 μm); this contributed to the lower volume percentage of particles with size below 100 nm in the filtered samples from the secondary treatment in Plant 1. This also occurred in some samples in Plant 2. The larger sized particles after filtration could potentially be attributed to two reasons. First, since the larger average particle size after filtration occurred only 19 times in Plant 1 (of those, 16 were ASP samples out of a total 143 samples) and only 5 times in Plant 2 (all were PE samples out of a total 130 samples), some filters might have been faulty and produced larger particles than intended. Another possibility could be that the particles after filtration interacted with each other and agglomerated into larger particles; however, we believe this to be less likely due to their inconsistent occurrence during the study.

Statistical analysis

As seen from the monthly trend, temperature and seasonality may have some influence on the size and removal efficiency of the particles during wastewater

TABLE 1 Pearson's correlation coefficients and their significance level among the parameters of unfiltered and filtered (through a 450-nm filter) supernatant particles in Plants 1 and 2.

	Plant 1				Plant 2			
	Unf CR	Unf Z_{avg}	Filtered CR	Filtered Z_{avg}	Unf CR	Unf Z_{avg}	Filtered CR	Filtered Z_{avg}
Unfiltered CR		0.8024**	0.7348**	0.6899**		0.4323**	0.5383**	0.3373*
Unfiltered Z_{avg}			0.5981**	0.7764**			0.0721	0.0477
Filtered CR				0.5840**				0.7375**
Filtered Z_{avg}								
Temperature	0.0948	0.0680	0.3871**	0.3326**	0.0340	−0.2314	0.2144	0.1226
Unfiltered COD	0.2934*	0.3203*	0.2182	0.2545	0.8877**	0.2233	0.3354*	0.2248
Filtered COD	0.2977*	0.2626*	0.2450*	0.3146**	0.8695**	0.1335	0.5551**	0.3717*
TSS	0.0575	0.0649	0.0482	−0.0032	0.5149**	0.2407	−0.4204**	−0.2121
VSS	0.0428	0.0603	0.0184	−0.0165	−0.4397**	0.2740	−0.3425*	−0.1635
Total EPS	−0.0285	0.0156	−0.0729	0.1459	0.3670**	−0.1078	0.4038**	0.3510*
TB-EPS	−0.0195	0.0597	−0.0711	0.0985	0.2289	−0.2071	0.3953**	−0.1390
LB-EPS	−0.0302	−0.1429	−0.0152	0.2123	0.4063**	0.2110	0.0932	0.4409**
EPS polysaccharide	0.0501	0.0201	−0.0091	0.0552	0.2105	−0.0304	0.5239**	0.4700**
EPS protein	−0.0572	0.0136	−0.0806	0.1610	0.3598*	−0.1150	0.3047*	0.2598
LB-EPS polysaccharide	0.0677	−0.0782	0.1216	0.0694	0.3644*	0.0418	0.3400*	0.1332
LB-EPS protein	−0.0880	−0.1272	−0.0874	0.2154	0.3722**	0.2143	0.0467	−0.1651
TB-EPS polysaccharide	0.0297	0.0587	−0.0680	0.0386	0.1460	−0.0394	0.5011**	0.4920**
TB-EPS protein	−0.0364	0.0489	−0.0595	0.1050	0.2266	−0.2300	0.3145*	0.3693**

Note: The highlighted coefficients were found to be significant at both plants.
*$p < 0.05$. **$p < 0.01$.

treatment. In addition, EPS, influent COD, TSS, and VSS may also have an influence on the particle size and abundance. Thus, we explored their relationship statistically using Pearson's correlation and PCA. Since augmentation was used, we performed a chi-squared test on the coefficients both with and without augmentation. The analysis showed that they were not significantly different from each other for both Plants 1 and 2, and so augmentation did not alter the PCA results significantly.

We used Pearson's correlation to assess the relationship between temperature and particle removal efficiency in Plants 1 and 2, and the only significant relationships were from Plant 1 (Table S5). The removal efficiency of the particles in the unfiltered samples for the entire Plant 1 had a significant inverse relationship with temperature. A similar and more significant relationship was observed for the removal of the particles in the unfiltered samples of the tertiary treatment, although, relative to the entire Plant 1, only a small percentage of unfiltered particles were actually removed during the tertiary treatment (Table S2). For the unfiltered samples, the removal

efficiency of the primary treatment, however, had a significant direct relationship with temperature. For the filtered samples, the removal efficiency in the secondary clarifier also had a significant and direct relationship. Thus, lower temperatures may help remove larger particles at Plant 1, but it may impact specific unit processes differently within the treatment plant.

Increasing temperature seemed to correlate with an increase of CR and Z_{avg} in filtered samples in Plant 1 (Table 1), but it did not correlate with other parameters nor any parameters from Plant 2. In addition, only a very slight correlation of temperature with CR and Z_{avg} was observed in the PCA for Plants 1 and 2 (Figure 3). This may be due to the relatively small seasonal temperature differences that Southern California experiences; thus, the result did not conclusively suggest that temperature had a substantial impact on the particle size and abundance though earlier visual observation of the data pointed to potential temperature effect.

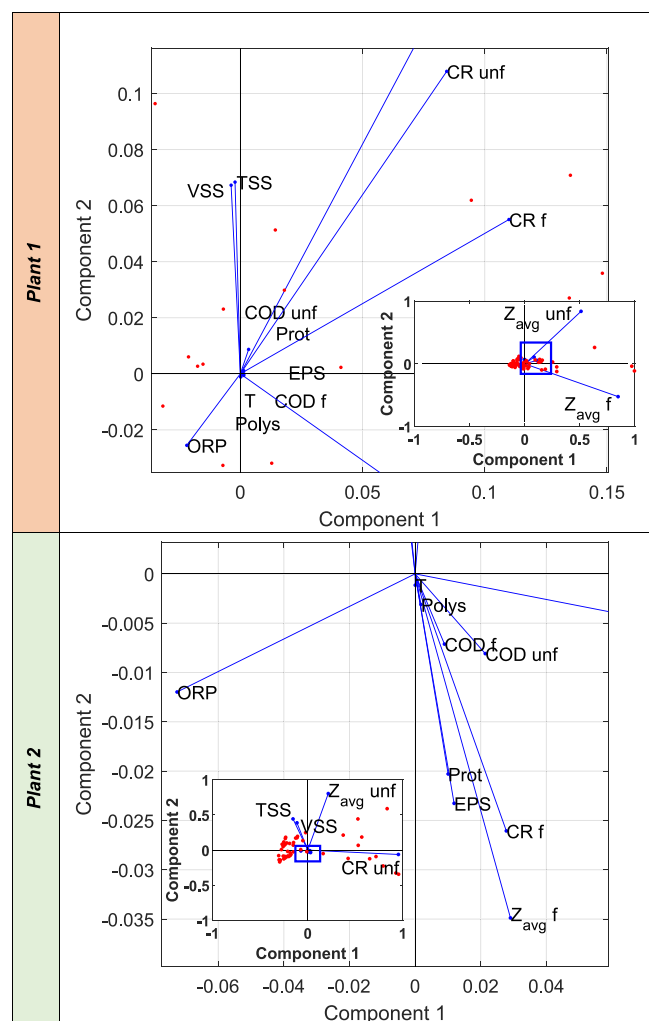Unfiltered COD seemed to correlate significantly with the abundance of unfiltered CR in both Plants 1 and

FIGURE 3 The principal component analysis (PCA) of selected parameters from Plants 1 and 2. Some parameters were augmented before being analyzed. The inset is the entire PCA of the parameters, and a portion of the inset details was enlarged in the graph. Abbreviations: COD, chemical oxygen demand; CR, count rate; EPS, extracellular polymeric substances; f, filtered; ORP, redox potential; Polys, polysaccharide; Prot, protein; T, temperature; TSS, total suspended solids; unf, unfiltered; VSS, volatile suspended solids; Zavg, average particle size.

2 (Table 1; Figure 3), while filtered COD in both Plants 1 and 2 correlated with both unfiltered and filtered CR and filtered Z_{avg}. This suggests that the availability of dissolved carbon source may impact the quantity of both the larger particles and nano-sized particles produced during the treatment process, and nano-sized particles tend to be bigger with higher carbon availability. The relationship between the increase of abundance of particles and the increase of dissolved carbon corroborates analyses above that the most suspended particles, especially nano-sized particles (Figure 2), were naturally occurring and generated biologically within the wastewater treatment

(Kowalkowski, 2010; Lead & Wilkinson, 2006), most likely by the microbes in the activated sludge. Subsequently, the results again suggest that the current WRRF processes are not efficient in removing nano-sized particles, and the increase of nano-sized particle abundance could also impact downstream water reuse treatment.

We also noted that filtered (i.e., dissolved) COD steadily became the dominating COD instead of particulate COD toward the end of the treatment (Figures S2 and S3). Larger particles contributed substantially to the total COD (unfiltered COD) in the primary treatment and the beginning of the secondary treatment (echoing unfiltered COD correlating significantly with the abundance of unfiltered CR; Table 1 and Figure 3), but the total and dissolved COD were quite similar by the end of the treatment ($COD_f/COD_{uf} \sim 1$) as the larger particles were removed by the treatment processes. This further suggests that larger particles were mostly biological in origin.

EPS and their components correlated significantly with both unfiltered and filtered CR and filtered Z_{avg} in Plant 2, but they did not correlate with any CR and Z_{avg} in Plant 1 (Table 1; Figure 3). This suggests that the production of EPS and their components contributes significantly to the quantity of both larger particles and nano-sized particles and the increase of the size of nano-sized particles in Plant 2. Since EPS components were found previously to vary with total inorganic nitrogen (Tseng et al., 2015) and the current study found that both large and nano-sized particles vary with dissolved carbon, operationally, WRRFs already monitoring dissolved carbon and nitrogen may be able to predict the abundance of larger and nano-sized particles during treatment to control or manage membrane fouling in downstream treatments. This warrants more studies on how dissolved carbon and EPS might generate particles. Managing dissolved carbon may help control the production of nano-sized particles in the wastewater treatment plants, with the goal of reducing cost in water reclamation and reuse in terms of energy use and damages to the membrane filters (Amy, 2008; Cogan et al., 2022; Jarusutthirak et al., 2002; Lee et al., 2005; Lesjean et al., 2005; Meng et al., 2006, 2009; Rosenberger et al., 2006; Safarik & Phipps, 2005).

The differences observed between the two plants may be attributed to the differences of the two plants' treatment processes and operation. Plant 1 was operating with an MLE with recirculation, which would render the flow in the biological treatment similar to a continuous stirred tank reactor (Tseng et al., 2015). In contrast, Plant 2 was operating with an LE with a flow resembling a plug flow. This may explain the significant correlation between EPS and their components with CR and Z_{avg} in Plant 2 but

not in Plant 1, since the recirculation in Plant 1 mixed the flow, it would be difficult to assess the relationship between EPS and CR or EPS and Z_{avg} in the biological treatment (Table 1; Figure 3). This may also explain the drastic decrease of CR and Z_{avg} during the biological treatment in Plant 2 but not as much change of CR and Z_{avg} in Plant 1 (Figure 2; Tables S1 and S2).

CONCLUSIONS

It is important to know the removal efficiency and the dynamic prevalence of nano-sized particles in WRRFs in order to accurately model and predict the production of nano-sized particles, examine their effect on subsequent treatment processes for water reuse, and investigate the fate and transport of certain contaminants associated with these particles. In this study, we found that secondary treatment was the most efficient treatment process in removing particles, suggesting that sedimentation is a major pathway for particle removal. Specifically, the two secondary treatment trains, TF and conventional ASP, were more efficient in removing particles larger than 450 nm than smaller particles (on average >88.9% removal efficiency of larger particles and 40.1–52.7% for nano-sized particles within the ASP treatment). In addition, we found that the conventional WRRFs are not designed to remove nano-sized particles efficiently due to the overall low removal efficiency of nano-sized particles (1.3–59.0%) and the increasing percentage of particles with size below 100 nm along the treatment train. The quantity of dissolved carbon (and EPS components in Plant 2) was found to significantly correlate with the quantity of both larger and nano-sized particles, suggesting that the majority of suspended particles in the wastewater treatment were generated biologically within the wastewater treatment; this result presents a contrast to the majority of the studies on nanoparticles in the wastewater focusing on the fate and transport of ENPs. This is also an opportunity because WRRFs can monitor dissolved carbon or nitrogen to, for example, control membrane fouling downstream. While cooler temperatures may aid the removal of larger particles in wastewater, the effect of temperature on the size and the quantity of these particles was less conclusive from our study, and this may be due to the small variation of temperature in Southern California. The results of this study contribute to a better understanding of fate and transport nano-sized particles in the wastewater, although the seasonality of the nano-sized particles variation was not conclusive, the results provided better treatment processes and control that can be implemented to manage nano-sized particles in wastewater.

AUTHOR CONTRIBUTIONS
D. Rosso, J. Smeraldi, and L. Y. Tseng contributed to the study conception and design. Material preparation, data collection, and analysis were performed by J. Smeraldi, L. Y. Tseng, and I. Dutta. Supervision of the project was performed by D. Rosso, L. Y. Tseng, and R. Ganesh. The first draft of the manuscript, including data visualization, was written by L. Y. Tseng, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

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