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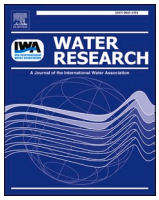

Wastewater analysis as a global toxicovigilance tool for the monitoring of new psychoactive substances

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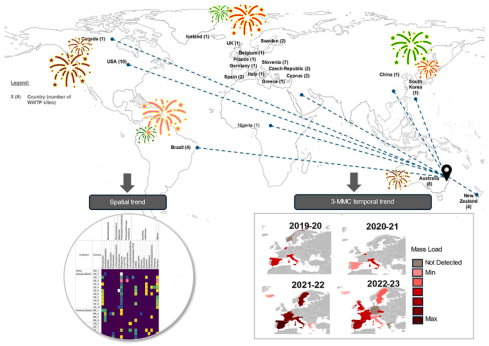
HIGHLIGHTS

- 21 new psychoactive substances (NPS) were found across 52 sites in 20 countries.
- The most frequently detected NPS was mitragynine, 3- MMC, and eutylone.
- 3,4-methylenedioxy PV8 was detected for the first time in sites in Australia and the US.
- A decline of 3-MMC was seen in European sites from 2021–2022 to 2022–2023.

GRAPHICAL ABSTRACT



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ABSTRACT

New psychoactive substances are a group of synthetic or naturally occurring drugs that mimic the effects of controlled illicit drugs. With limited information around potency, effects and health risks, there is international concern around their use and thus surveillance efforts are needed for public health. This study presents a global assessment of new psychoactive substances in influent wastewater from 52 sites across 20 countries during the 2022/2023 New Year period. Using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry, 21 new psychoactive substances were detected with mitragynine, 3-methylmethcathinone, and eutylone being the most prevalent. Notably, 3,4-methylenedioxy-PV8 was identified for the first time in sites in Australia and the United States. A retrospective analysis of population normalised mass loads for 3-methylmethcathinone at European sites revealed downward trends in 2022/2023 sampling period compared to the previous years, suggesting a possible impact of regional scheduling measures. Additionally, this work demonstrates the stability of new psychoactive substances in loaded cartridges for up to four months when stored at -20 °C. These findings highlight the value of wastewater-based epidemiology for global monitoring of emerging new psychoactive substances threats and policy evaluation.

Introduction

New psychoactive substances (NPS) are a group of synthetic or naturally occurring chemicals that mimic the effects of controlled illicit drugs but are not listed in the United Nations International Drug Convention 1961–1971 (United Nations Office on Drugs and Crime, 2024). These substances are not necessarily 'new'. Some, such as benzimidazole synthetic opioids, were synthesized in the 1950s and never intended for human consumption (Ujváry et al., 2021). New chemicals have continued to circumvent the early warning systems replacing illicit drugs and scheduled NPS in the drug market (European Monitoring Centre for Drugs and Drug 2021). As of 2024, the United Nations Office on Drugs and Crime (UNODC) have identified 1300 NPS (United Nations Office on Drugs and Crime, 2023), while the European Union Drug Agency (EUDA) (European Union Drug Agency, 2024) were monitoring over 1000 NPS, with 47 new substances emerging in European market during 2024 (European Union Drugs Agency 2024). In October 2024, 83 NPS were placed under international control (United Nations Office on Drugs and Crime, 2023), underscoring the continuing global need for regulation informed by toxicological risk assessments related to human consumption.

The fatalities due to toxicity and increased recreational use of NPS has been well documented (Kronstrand et al., 2018; Andreasen et al., 2015; Nugteren-van Lonkhuyzen et al., 2022; Montanari et al., 2022). Given the dynamic nature of the drug market and the sporadic emergence of new compounds, a range of complementary tools are employed to monitor NPS occurrence. These include drug seizure records (Vincenti et al., 2021), clinical toxicology reports, forensic post-mortems, population surveys (12), dark-web surveillance (Catalani et al., 2021), and wastewater-based epidemiology (WBE) (Borova et al., 2015; Castiglioni et al., 2021). Some countries have also implemented early warning systems (EWS) to rapidly detect and respond to emerging NPS threats, enhancing coordination across forensic and public health sectors (Evans-Brown and Sedefov, 2018). Each method serves a distinct role and offers insight at different stages with its uncertainty, ranging from early detection and tracking of consumption patterns to assessing health outcomes and regulatory action.

WBE is recognised for its near real-time reporting of population-level sewage biomarkers through anonymised, aggregate samples collected from wastewater treatment plants (Daughton, 2018). Following ingestion, exogenous compounds are excreted in urine and faeces either unchanged as parent compounds or as metabolites. The concentration of these biomarkers in raw wastewater can be used to estimate population-level consumption by back calculating based on the catchment population size and flow rate data (Daughton, 2001; Daughton, 2011). However, with limited pharmacokinetic data available for NPS, accurate back calculation requires cautious interpretation. Nevertheless, WBE has provided valuable insight for public health, which was demonstrated during the Covid-19 outbreak, where population-level monitoring was achieved in real time without recruiting individuals and thus avoided human ethics and logistic procedures (Bivins et al., 2020).

In 2019, an international NPS wastewater monitoring was initiated to provide insight into NPS use during the New Year's period (Bade et al., 2021). A similar study was also conducted in Europe, comparing pooled weekdays and weekends for weekly use patterns (Castiglioni

et al., 2021). Other NPS studies have offered country-specific insights (Salgueiro-González et al., 2022; Jaunay et al., 2024; O'Rourke and Subedi, 2020; Gao et al., 2017; Senta et al., 2015; Bade et al., 2020) or focus on specific events (Bijlsma et al., 2020; Nadarajan et al., 2024), collectively demonstrating the feasibility of WBE as the complementary tool for near real time analysis.

The regulation of NPS varies globally. Some countries have broad definitions, implementing blanket bans or total prohibition of all NPS, while others use more targeted approaches, scheduling NPS based on their structural scaffolds (analogue control), or through individual listing (Evans-Brown et al., 2022). NPS may also be regulated under customs acts, medicinal legislation, and/or through consumer law, depending on national context. Among the NPS, 3-methylmethcathinone (3-MMC) has consistently been one of the most frequently detected NPS in all iterations of the international wastewater project since its inception in 2019/2020 (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). It has also been one of the most frequently reported NPS to the European Union Early Warning System, with notable rises in customs seizures recorded in 2020 (European Union Drug Monitoring, EU Drug Market, 2024).

Due to the potential for problematic patterns of use, efforts were made to include 3-MMC under international control (UNODC 2024). Although it was not scheduled following the initial UNODC critical review in 2016, growing evidence from customs seizures and toxicology cases prompted a second review in 2022 (World Health Organisation 2022). Eventually, in March 2023, 3-MMC was formally placed under international control, alongside six other NPS, following the recommendation by the World Health Organisation (WHO) expert committee (UNODC 2022). It is now classified as Schedule II Drug under the Convention of Psychotropic Substances of 1971 and entered into force in November 2023.

While the list of NPS from EUDA is similar to UNODC, regulatory actions within European Union may precede the international scheduling. For example, 3-MMC was added to the EU's list of controlled drugs on 18 March 2022, a year before the UNODC, through Commission Delegated Directive (EU) 2022/1326 (Commission, 2022). The member states were given a year until 18 February 2023 to adopt the directives into their national legislation. Before this directive, 15 Member States controlled 3-MMC under national drug control legislation, six under NPS legislation, and one under other legislation.

The New Year period is recognised as a time of the year of potentially increased recreational drug use (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). The overarching aim of this study is to investigate global NPS use during 2022/2023 New Year's period. The specific objectives were: (i) to determine the geographical prevalence of NPS use during this period; and (ii) to assess the potential impact of 3-MMC legislation by analysing longitudinal wastewater data in the context of national and international scheduling. In addition to the specific objectives, the study also included an assessment of stability of NPS on loaded solid phase extraction (SPE) cartridges to support reliable sample shipment and analysis. Materials and Methods

2.1. Selection of NPS

This work investigates 54 NPS (*Table S1–1, Electronic Supplementary Information 1)*. They were selected based on data published by UNODC Early Warning Advisory (United Nations Office on Drugs and Crime, 2023), EUDA Early Warning System (European Union Drug Agency, 2024), and in verbal consultation with local and international forensic agencies and are thus all of public health relevance.

2.2. Chemicals and reagents

Analytical reference standards used for quantification at The University of Queensland were kindly donated by Forensic Science Queensland, Australia. Deuterated internal standards (ISTD) benzoylecgonine-d3, ketamine-d4, 3,4-methylenedioxymethamphetamine (MDMA)-d5, mephedrone-d3, methylone-d3, and *O*-desmethyl-cis-tramadol-d6 were purchased from Cerilliant (Round Rock, TX, USA) and morphine-d3 monohydrate from Lipomed GmbH (Germany). Formic acid (Optima LC/MS grade, purity ≥99.0 %), isopropanol, dichloromethane, glacial acetic acid, and ammonia (28 %) were from Fisher Scientific (Tingalpa, QLD, Australia). LC/MS hypergrade methanol (MeOH, purity ≥99.9 %) and hydrochloric acid (HCl fuming 37 %) were obtained from Merck (Highway Bayswater, Australia). Working standards consisting of 54 analytes at 0.05 ng/mL, 5 ng/mL, and 50 ng/mL in MeOH were prepared along with mixed ISTD at 12.5 ng/mL in MeOH. All standards were stored in amber vials at −20 °C until use. Filtered ultrapure water (resistivity 18.2 MΩ cm at 25 °C) was from a Milli-Q system (Millipore, Bedford, USA).

2.3. Sample collection

Influent wastewater samples were collected from three to 11 days from the 25th of December 2022 to the 5th of January 2023. For the majority of sites, they were collected for between 7 and 11 consecutive days (Table SI-2). However, in one site in the United Kingdom, samples were collected on seven non-consecutive days between 11th to 25th December 2022 while for eight sites in the United States, samples were collected for three non-consecutive days between 27th December 2022 to 4th Jan 2023. In total, samples were collected from 52 wastewater treatment plants in 20 countries: Australia (two-letter ISO code: AU, $n =$ 8 sites), Belgium (BE; 1), Brazil (BR; 4), Canada (CA; 1), China (CN; 1), Cyprus (CY; 2), Czechia (CZ; 2), France (FR; 1), Germany (DE; 1), Greece (GR; 1), Iceland (IS; 1), Italy (IT; 1), Republic of Korea (KR; 1), New Zealand (NZ; 4), Nigeria (NG; 1), Slovenia (SI; 7), Spain (ES; 2), Sweden (SE; 2), United Kingdom (UK; 1), and the United States (US; 10). These sites covered about 20 million inhabitants from both small and large cities as well as holiday destinations. The sampling date, flow rate, and population size are reported in *Table S1–2, Electronic Supplementary Information 1*. A standard protocol for the collection procedure was provided to all collaborators to minimise variability. Briefly, 24-hour composite samples were collected by flow or time proportion. The collected samples were acidified immediately to pH 2 using 2 M hydrochloric acid to suppress microbial growth, then stored at −20 °C until SPE sample extraction.

2.4. SPE extraction and validation

The sample treatment followed an SPE protocol (Bade et al., 2020) distributed to all participants using UCT XtracT DAU, 500 mg/ 6 mL cartridges (UCT Inc, Bristol, PA, USA). Steps 1–7 (*Text S1. Supplementary Methodology - 1. SPE Extraction, Electronic Supplementary Information 1*) were carried out in the country of collection. The ISTD were added after filtration and pH adjustment in Step 3 at the country of origin except for samples from Germany, Greece, and United States Site 2. After washing, the cartridges were dried, stored at −20 °C (Step 7), and shipped to The University of Queensland for elution and LC-MS/MS analysis (Steps 8 to 12).

The SPE method in this work was an adapted method with the addition of new compounds (*Table S1–3*) and has been validated previously (Laimou-Geraniou et al., 2025). The method validation includes selectivity, recovery, matrix effect, limits of detection (LOD), and limits of quantification (LOQ) in wastewater. The linearity and *R*-squared values were assessed using a 10 % MeOH solution. In addition to the validation, loaded cartridges were examined for NPS stability under various temperatures and storage conditions. The details for the validation procedure and cartridge stability are included in *Electronic Supplementary Information 1* in Text S1: Supplementary Methodology, Section 2 (SPE validation) and 3 (Stability of loaded cartridges), respectively.

2.5. HPLC-MS/MS

The instrument was an LC (Shimadzu Nexera LC-40, Kyoto, Japan) coupled to a tandem mass spectrometry instrument (SCIEX Triple Quad 7500 system, Framingham, MA, USA) (Nadarajan et al., 2024). *Table S1–4* details the operating conditions for liquid chromatography and mass spectrometry. All data were acquired in positive ionisation with scheduled multiple reaction monitoring (sMRM) mode as shown in *Table S1–1*.

2.6. Quantification and quality control

The identification and confirmation of NPS compounds followed N° SANTE/11,312/2021 (40) guidelines based on the two product ion transitions with an ion ratio of ± 30 % and a retention time of ± 0.1 min of a reference standard. The concentration of NPS compounds was determined using a nine-point external calibration curve (0.5 – 1000 ng/L) prepared in 10 % MeOH containing ISTD. Quantification of unknown was based on the peak area ratio between known analyte in the calibration curve and ISTD. Sites without ISTD were reported qualitatively as 'Detected'. Procedural blanks were analysed at the beginning of each sample site. To confirm the identity of NPS detected for the first time in wastewater, reference standards were spiked into SPE extracts and compared with their respective unspiked counterparts. Instrumental blanks and quality control standards were sequenced after every ten samples to monitor the batch performance. All reported concentrations were procedural blank-corrected, whenever needed.

The population normalised mass load (PNML) was estimated based on Eq. (1). The concentration (ng/L) obtained from the sample was multiplied by the daily influent wastewater flow rate (Megalitre, ML) for the population served in the catchment, normalised to 1000 people. In this work, the specific correction factor on parent/metabolite ratio was not included in the back-calculation due to limited information on NPS pharmacokinetic studies.

$$\text{Population normalised mass load (PNML, mg / day / 1000 people)} = \text{concentration}\left(\frac{\text{ng}}{\text{L}}\right) \times \text{flow rate (ML)} \times \frac{1000}{\text{population}} \quad (1)$$

2.7. Statistical method and data analysis

For the 3-MMC trend analysis, additional statistics were performed. The normality of the distribution for temporal data was tested using the Shapiro-Wilk test and the Kolmogorov-Smirnov test. Due to the non-normal distribution of the data, Kruskal-Wallis's test was applied to check the significance of the temporal trend of the 3-MMC PNML. The mean rank contribution of results within the groups was statistically compared pairwise using post-hoc Dunn's test. For the number of sites $n = 2$, the Mann-Whitney U test was applied to check the null hypothesis. For all results, the null hypothesis was not rejected when the p-value > 0.05 at two-tailed. Sites with $n \leq 2$ were compared visually using descriptive statistics.

Peak integration was done using SCIEX OS. Statistical analysis and visualisation were performed using Microsoft Excel Version 2410 and GraphPad Prism Version 10.1.2 for Windows (Boston, MA, USA).

Results and discussion

3.1. SPE method validation

The SPE method was validated and reported previously for selectivity, limit of detection (LOD), limit of quantification (LOQ), recovery, and matrix effects (Laimou-Geraniou et al., 2025). The analytical figures of merit are provided in *Table S1–5 in Electronic Supplementary Information 1*. The LOQ ranged between 0.29 ng/L and 12 ng/L across all compounds. Most NPS exhibited SPE recoveries between 80 % and 120 %. Acetoxymethylketobemidone, clonazolam, flutoprazepam, phenibut, and SL-164 had recoveries below 50 % at 10 ng/L and 100 ng/L. Meanwhile, flubromazolam recovered at 121 % ± 3.2 % at 10 ng/L and 194 % ± 3.8 % at 100 ng/L. The matrix effect was substantial, with ion suppression predominating in the SPE matrix. Thirty compounds at 10 ng/L and 19 compounds at 100 ng/L exhibited a matrix effect of ± 51 %. Due to the unavailability of isotopically labelled ISTD for most target NPS, MDMA-d5 was employed as a surrogate ISTD for quantification.

3.2. Stability of analytes in loaded SPE cartridges

One critical aspect of this study was assessing the stability of NPS on SPE cartridges, particularly to ensure the analytes remain stable during international shipping and potential delays. As such, an experiment was conducted to test the stability of all analytes spiked at 500 ng/L in influent wastewater and loaded onto SPE cartridges. As the standard protocol involves shipping dried SPE cartridges to Australia, the stability study commenced after loading and drying steps. A total of 61 analytes, including the seven ISTD, were evaluated for stability at five temperatures (−80 °C, −20 °C, 4 °C, 22 °C, and 37 °C) across five time points (three days to four months), simulating differing storage conditions, as well as the winter and summer conditions over the New Year period around the world. Stability was considered acceptable with recoveries remaining between 80 % and 120 %. Out of 54 compounds, two NPS (phenibut, and acetoxymethylketobemidone) had recovery below 80 % and six (isotonitazene, etonitazepyne, 25iP-NBOMe, protonitazene, N-desethyl isotonitazene, butonitazene, and desoxymethoxetamine) had recovery above 120 % at −20 °C up to four months storage. Data were unavailable for the three-day and one-month time points at −20 °C and day three at 22 °C due to sample loss. Individual stability profiles for each analyte are presented *in Fig. S1–1 in Electronic Supplementary Information 1*.

These findings provide a foundation for future monitoring studies of current and emerging NPS and their stability on cartridge. For example, the nitazene compounds exhibited slightly more variation in their stability over time. Nevertheless, based on our study, storage at −20 °C is ideal, along with prompt shipment of cartridges within four months and immediate analysis of extracts after extraction to minimise the degradation of sensitive NPS.

3.3. 2022/2023 NPS snapshot

A summary of the 2022/2023 New Year campaign conducted across 52 sites from 20 countries using 343 samples is presented in Figs. 1 and 2. Based on Fig. 2, the most prevalent NPS classes in this cohort were synthetic cathinones (42 %), followed by benzodiazepines (23 %), phencyclidine-type substances (14 %) and phenmetrazines, nitazenes,

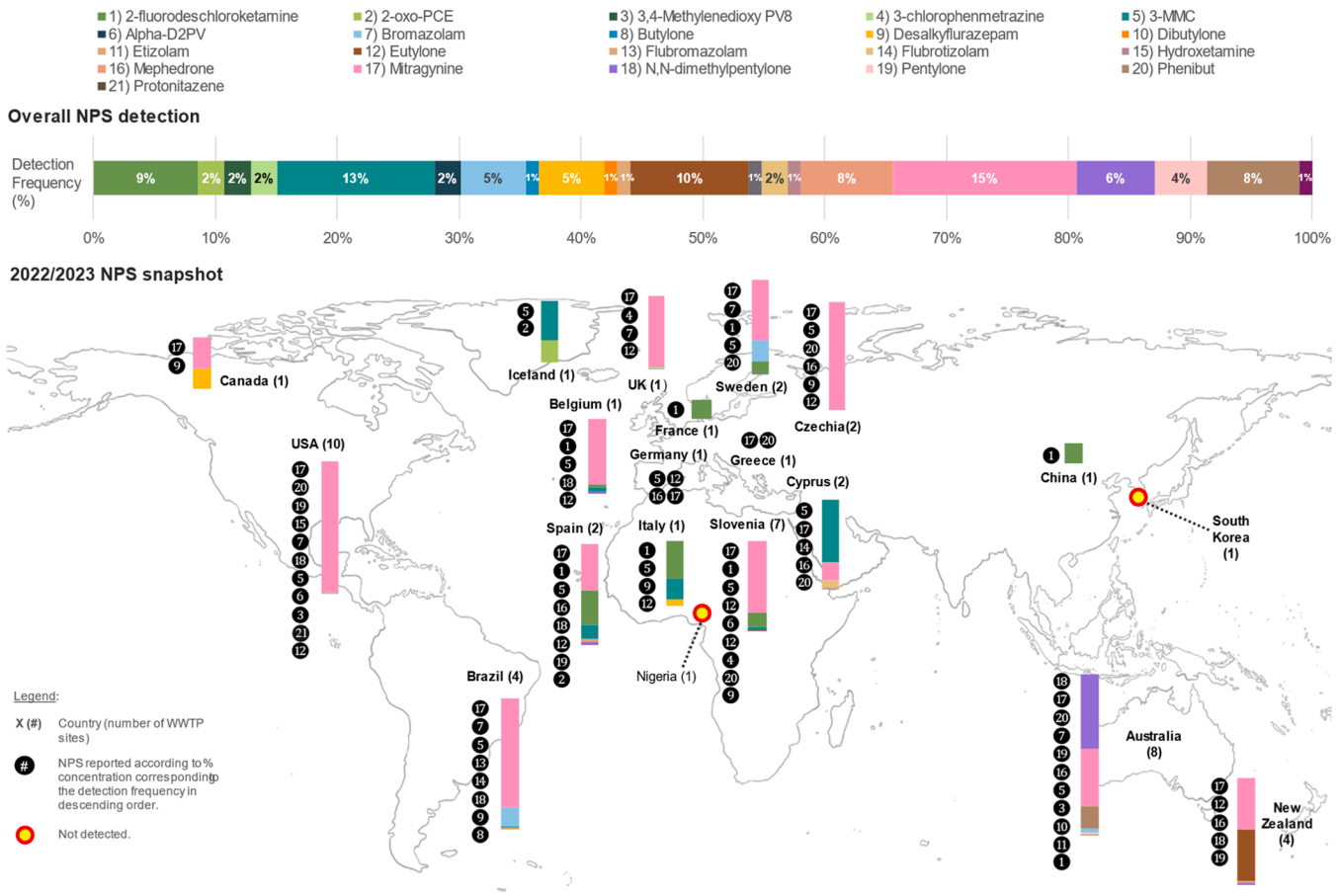

Fig. 1. Summary of detected NPS across sampling sites, including their respective detection frequencies. Note: PNML estimates are based on individual WWTP catchments and should not be extrapolated to entire cities or countries.

plant-based and others, each at 4.8 %. Of the 54 NPS in the method, 21 were detected: 2-fluorodeschloroketamine (2F-DCK), 2-oxo-PCE, 3,4-methylenedioxy PV8, 3-chlorophenmetrazine, 3-methylmethcathinone, alpha-D2PV, bromazolam, butylone, desalkylflurazepam, dibutylone, etizolam, eutylone, flubromazolam, flubrotizolam, hydroxetamine, mephedrone, mitragynine, *N,N*-dimethylpentylone, pentylone, phenibut, and protonitazene. The chemical and structural information is provided in *Table S1–6*. The most frequently detected NPS was mitragynine (38/55 sites), followed by 3-MMC (28/55) and eutylone (25/55), which is consistent with previous work (Bade et al., 2023). Compounds such as flubromazolam and butylone were only found in sites in Brazil, whereas protonitazene and hydroxetamine were only detected in sites in the United States. Although methcathinone and *O*-desmethyltramadol were detected at high concentrations in all sites in this work, they were excluded from the discussion, due to the uncertainty around their consumption. Methcathinone is potentially an in-sewer oxidation product of ephedrine or pseudoephedrine (Simpson et al., 2022), while *O*-desmethyltramadol is a metabolite of tramadol. However, it has also been sold and used as an NPS on its own (Castro et al., 2022). Therefore, this study could not distinguish between the licit and illicit use of tramadol and its metabolite, *O*-desmethyltramadol. No NPS were detected in samples from Nigeria and the Republic of Korea. However, previous studies in Korea have reported the presence of *N*-ethyl pentylone and methcathinone, phenmetrazine, 25E-NBOMe, 25D-NBOMe, N-methyl-2-AI, *N*-ethylpentylone, etizolam, and eutylone (Bade et al., 2022; Bade et al., 2023; Kim et al., 2023; Lee and Oh, 2023). Although both local and international media have reported the use of plant-based and non-NPS substances in Nigeria, a 2021 review emphasised that empirical research on NPS in the country remain scarce (Dumbili et al., 2021). The current method includes limited number of plant-based target analytes, which may restrict the detection of such compounds. The PNML and the detection frequency for each NPS and site are provided in *Electronic Supplementary Information 2*.

3.3.1. *Synthetic cathinones*

As shown in *Fig. 2*, synthetic cathinones were the most detected class of NPS in this work. Among the cathinones, 3-MMC was the most frequently found, particularly in Europe, where it was detected in all but two sites. It was also present in samples from the United States and Australia. Mephedrone and eutylone were the next most prevalent cathinones, with the highest PNML levels observed in sites in New Zealand. Both compounds were also detected at lower levels in sites across Europe and the United States. Mephedrone is an isomer of 3-MMC, the current chromatographic method was able to differentiate between the two compounds (*Fig. S1–2*).

Although mephedrone has been prohibited in Europe since 2010, it was consistently detected between 2019 and 2022 (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). In the present study, mephedrone was detected in five European sites: Cyprus (CY1), Czechia(CZ1), Germany, Slovenia (SI4), Spain (ES1), and the United Kingdom with the highest PNML of 4.3 mg/day/1000 people observed in the United Kingdom on 18th December 2022. It is also detected at two sites in Australia and one in New Zealand.

As shown in *Table S1–6,* eutylone, a structural isomer of pentylone, was detected in Belgium, Czechia, Germany, Italy, New Zealand, Slovenia, Spain, the United Kingdom and the United States. The highest PNML recorded in this study was in a site in New Zealand (653 mg/day/1000 people), marking the highest level detected since monitoring began in this international study (Bade et al., 2022; Bade et al., 2023).

N,N-dimethylpentylone and pentylone (also a metabolite of *N,N*-

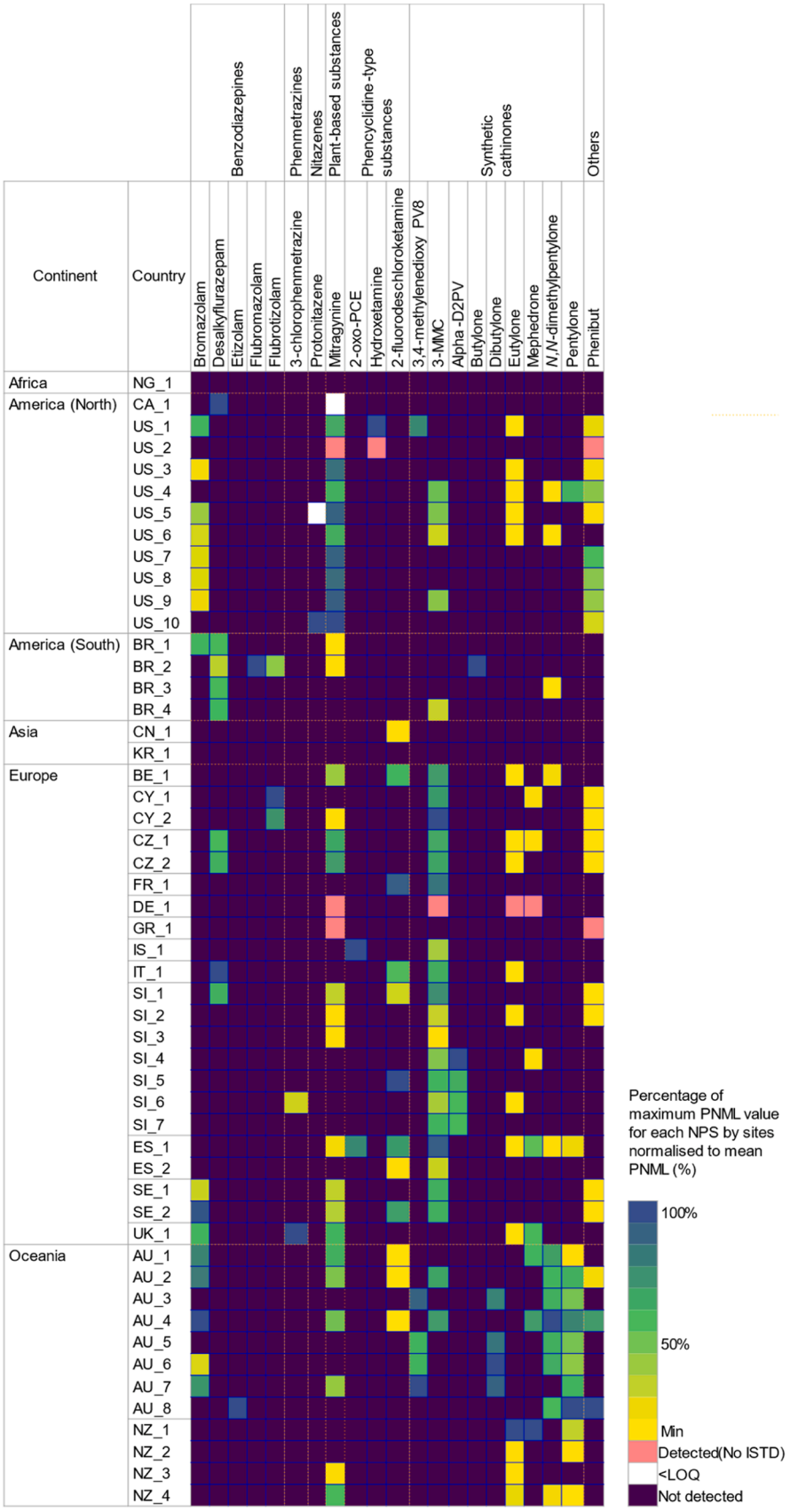

Fig. 2. Heat map showing percentage of maximum PNML for NPS detected at 52 sites.

dimethylpentylone) (Jaunay et al., 2024), were found almost exclusively in Oceania, appearing across sites in Australia and New Zealand. The highest PNML for *N,N*-dimethylpentylone, 63 mg/day/1000 people, was observed at site AU7 on 3rd January 2023. The highest for pentylone was 15 mg/day/1000 people at site AU8 on 2nd of January 2023.

In this work, one synthetic cathinone, 3,4-methylenedioxy PV8, was detected for the first time at sites in Australia and the United States. To confirm its identification via LC-MS/MS, a secondary experiment was conducted, in which the extract was spiked with a reference standard (*Fig. S1–3*), confirming the presence of the compound at a retention time of 5.1 min. 3,4-Methylenedioxy PV8 was detected at four sites in Australia (AU3, AU5, AU6 and AU7) throughout the entire sampling week, with increased levels observed over the New Year period, reaching a maximum PNML of 0.98 mg/day/1000 people. In the United States, 3,4-methylenedioxy PV8 was detected at one site, albeit at much lower levels. The first 3,4-methylenedioxy PV8 clinical case was reported in Sweden in 2019 through a retrospective analysis of oral fluid samples (Axelsson et al., 2022). In September of the same year, the compound was formally notified to the EMCDDA (now the EUDA). Subsequently, in May 2020, a surveillance tool based on web crawling known as NPSfinder® reported the online discussion of the same compound on social media (Catalani et al., 2021).

3.3.2. *Designer benzodiazepines*

Three different classes of designer benzodiazepines were detected in the 2022/2023 New Year period: 1,4-benzodiazepin-2-one (desalkylflurazepam), thienotriazolobenzodiazepine (flubrotizolam), and triazolobenzodiazepine (bromazolam and flubromazolam). The highest PNML values were observed for bromazolam (48 mg/day/1000 people) in Sweden, desalkylflurazepam (4.2 mg/day/1000 people) in Canada, flubrotizolam (2.3 mg/day/1000 people) in Cyprus, and etizolam (0.33 mg/day/1000 people) in Australia.

Bromazolam was detected at five sites in Australia, one in Brazil, two in Sweden, one in the United Kingdom, and six in the United States. First reported in Sweden in 2016 (WHO 2023), bromazolam continues to be detected in the wastewater samples. Desalkylflurazepam (known as norflurazepam) (Manchester et al., 2018) was detected in sites in Canada, Brazil (BR1- BR4), Czechia (CZ1 - CZ2), Italy, and Slovenia (SL1). It is both an active metabolite and structural analogue of several benzodiazepines, including flurazepam, flutoprazepam, fludiazepam, midazolam, flutazolam, quazepam, and ethyl loflazepate (Manchester et al., 2018; Miyaguchi et al., 2006). Flubrotizolam was detected in Brazil (BR2) and Cyprus (CY1 and CY2), whereas flubromazolam was detected only at site BR2 in Brazil. Due to the high recovery of flubromazolam during method validation, the result should be interpreted cautiously.

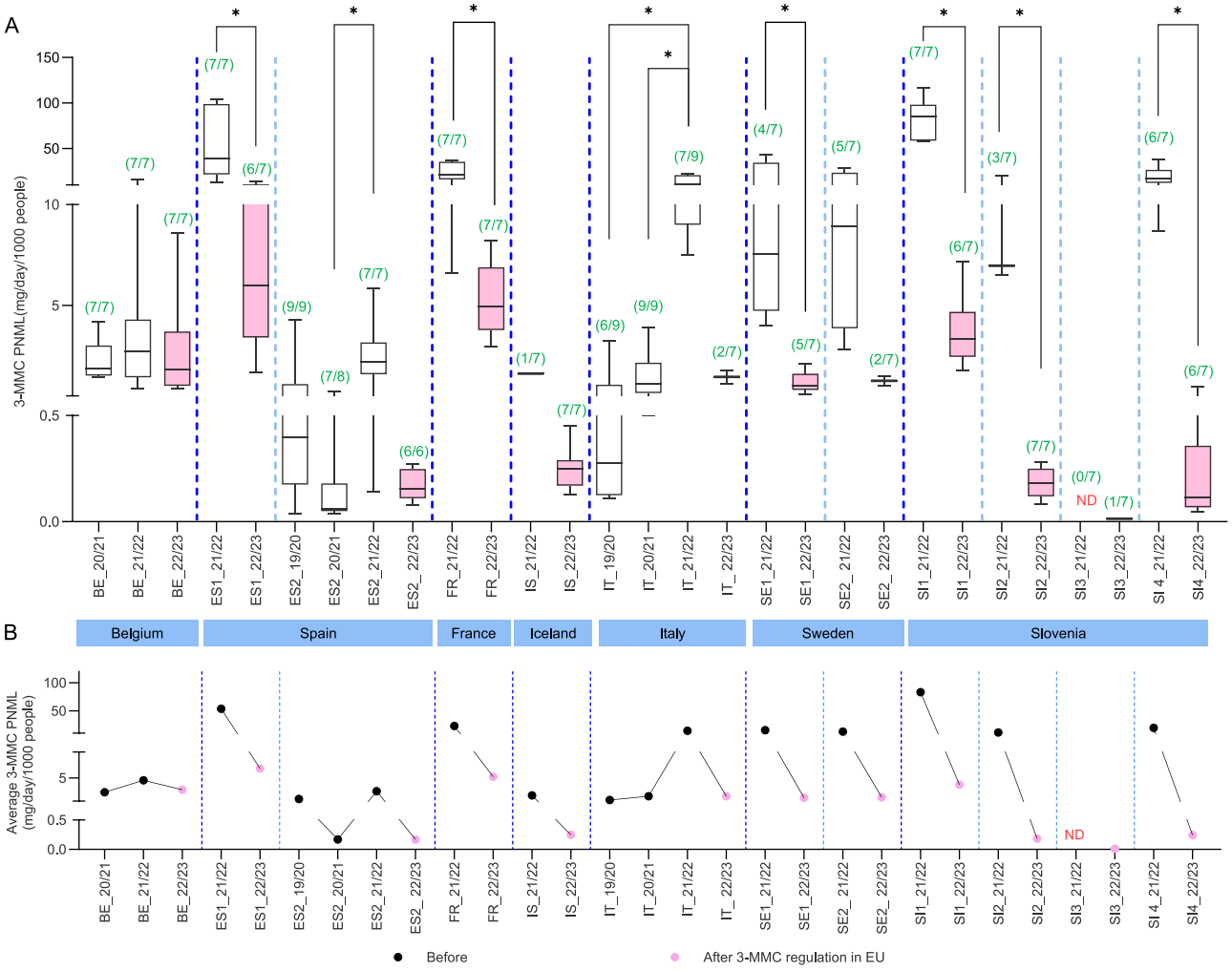

Fig. 3. A) Population normalised mass loads (PNML) for 3-MMC at 12 European sites in this study from 2019/20 to 2022/23. Statistically significant differences ($p < 0.05$) are indicated with a star (*). Numbers in green with brackets represent the detection frequency (number of days 3-MMC detected / total sampling days). 'ND' in red denotes 'not detected'. Pink bars represent PNML calculated in this study (i.e. 2022/2023) during the introduction of 3-MMC legislation in EU. The dark and light blue dash lines differentiate between countries and the sites within country, respectively.
B) Summary of trend before and after the implementation of Commission Delegated Directive (EU) 2022/1326 in March 2022. Black circles (pre-implementation); pink circle: after implementation.

3.3.3. Other compounds

Mitragynine was the most frequently detected NPS in this study and also exhibited the highest PNML among all the NPS in this work. However, as it remains legal in some countries, including all states in the United States where samples were collected, distinguishing between licit and illicit use remains challenging. The synthetic opioid protonitazene was detected in two sites in the United States, as previously published (Bade et al., 2024). 3-Chlorophenmetrazine was found at two sites in Europe (Slovenia and United Kingdom). Although phenibut is not classified as an NPS under the UNODC definition, it was detected across sites in the United States, Europe and Oceania. Three ketamine analogues were also identified: hydroxetamine, 2-oxo PCE and 2F-DCK, with 2F-DCK being the most frequently seen in sites in Australia, Belgium, China, France, Italy, Spain, Sweden and Slovenia. The highest PNML for 2F-DCK was recorded in Australia at the site AU4 with PNML of 72 mg/day/1000 people.

3.4. Temporal trend of 3-MMC 2019/2020 – 2022/2023

A temporal trend analysis for 3-MMC was conducted across multiple years with a focus on European sites located in Belgium, Spain, France, Iceland, Italy, Sweden, and Slovenia. This region was selected based on the EUDA 3-MMC scheduling decision in 2022 and the availability of longitudinal wastewater data from 2019/2020 and 2021/2022 (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). As shown in *Fig. 3*, significant trends in 3-MMC were observed in Europe. Belgium (BE) was the only site that showed no changes in PNML across the three sampling years from 2020/2021 to 2022/2023.

Sites in Spain (ES1 and ES2), France, Iceland, Italy, Sweden (SE1 and SE2), and Slovenia (SI1- SI4) had high 3-MMC PNML during 2021/2022 compared to the preceding and/or subsequent years. A Kruskal-Wallis's test showed significant differences across sampling years at the Spanish site ES2 (H (3) = 13.89, p = 0.003). Post hoc Dunn's test indicated significant increase from 2020/2021 and 2021/2022 (p = 0.002) at the same site. Similarly, in Italy, an increase was observed in 2021/2022 compared to preceding years. Both sites showed a statistically significant increase of 3-MMC PNML during 2021/2022 in conjunction with Covid-19 pandemic. A subsequent decline was observed at all sites in the following year (present study) except in Belgium, Spain (ES2), and Slovenia (SI3). Although 3-MMC appears to persist in some sites, none of the mass loads have reached the heights of 2021–2022.

The mass loads in this study were consistent with the wastewater data reported for 3-MMC in Europe from 2019 to 2021. For example, multiday pooled wastewater samples collected on weekdays and weekends in March and April 2021 ranged from 0.91 to 8.50 mg/day/1000 people in two Belgian cities (Salgueiro-Gonzalez et al., 2024). The three years New Year results showed a similar mass load range, between 0.87 to 16.15 mg/day/1000 people, indicating relatively stable use of 3-MMC in Belgium, which may not be influenced by festival activities. Additionally, studies conducted in Italy show similar trends to our work. The multiday pooled weekends mass loads increased from 0.12 – 3.80 mg/day/1000 people in 2020 (Salgueiro-González et al., 2022) to 3.64 to 6.99 mg/day/1000 people in 2021 (Salgueiro-Gonzalez et al., 2024).

Other data sources corroborate these increases in 3-MMC observed in sites in Europe. Postal seizures in Italy between May and October 2020 show that 3-MMC was the most seized NPS (Vincenti et al., 2021). A sharp increase was also observed in 3-MMC poisonings reported by healthcare professionals to the Dutch Poisons Information Centre in the Netherlands from 2019 to the first half of 2021 (Nugteren-van Lonkhuyzen et al., 2022).

Official reports from the European Union have identified synthetic cathinones, including 3-MMC, being sold as "legal" alternatives of MDMA or other stimulants (Commission, 2022). This is supported by drug-checking services across 13 European sites, which reported synthetic cathinones as the most common adulterants found in MDMA samples (EUDA 2024). However, adulteration with 3-MMC was not specifically mentioned in their report. Since the scheduling of mephedrone, 3-MMC has replaced other cathinones in the European market (Ferreira et al., 2019; Bäckberg et al., 2015). To further understand the relationship between 3-MMC and MDMA, wastewater data from 2019 to 2023 was analysed for spatial trends for Belgium, Italy, Spain, Slovenia, and Sweden using the EUDA/ SCORE dashboard data (EUDA 2024). A lower consumption of MDMA was observed at all sites for the year 2021/2022 compared to 2020/2021 and 2022/2023 (*Fig. S1–4*). While wastewater data alone cannot definitively show compound substitution and/or adulteration, it is worth noting that the observed trends align with the findings of the EUDA.

Overall, based on the four years data, a decline population-normalised mass loads were observed at sites in Spain and Italy in 2022/2023 compared to the previous years, except for Belgium. A general declining trend was observed at sites in Iceland, France, Slovenia, and Sweden. This coincides with legislation being introduced in Europe and internationally to control 3-MMC. It is potentially too early to confirm this trend is directly associated with the legislation, as there could be multiple factors at play. Hence, long term continuous monitoring is recommended to understand the trend and effectiveness of these control measures.

3.5. Challenges in the present work

The present work demonstrates the application of a wastewater analysis workflow on a global scale to understand the spatial-temporal trend of NPS, covering a population of over 20 million people across 20 countries for the 2022/2023 New Year period. However, it must be noted that the sites within this study cannot reflect the entire country. The method targeted 54 NPS, representing, <5 % of the over 1300 known NPS reported to date. Therefore, the observed prevalence is inherently method-dependent and does not imply the absence of other NPS in the sites and countries monitored. High-resolution mass spectrometry may serve as a complementary approach, provided the instrument's sensitivity is sufficient to detect trace levels of NPS (Bijlsma et al., 2021). Moreover, non-detections of compounds within this study do not mean that these compounds were not used, considering the different dose sizes of the NPS and the potential small user pool.

This study focused exclusively on samples collected during the New Year period, a time specifically selected as there will be typically increased drug use associated with festive occasions. However, this means that the levels of NPS observed in the current work may be higher than those normally seen in 'normal' weeks, and care should be taken when comparing studies. Additionally, it is important to recognise that data from a single site does not represent the entire country. Nevertheless, detection of an NPS at a single location may still indicate localised use, which could have public health implications. For example, the detection of protonitazene, a potent synthetic opioid, at even one site may warrant serious attention.

Finally, a recognised uncertainty in wastewater-based epidemiology is the estimation of the catchment population. The New Year period typically involves population movement due to holidays and travel (Boogaerts et al., 2024). This could skew the data towards potentially higher consumption trends in the regions where the occasion is celebrated. As such, there may be a larger than normal uncertainty associated with the population used due to holiday travel. In this study, many sites used static population estimates based on census data across sampling weeks, which may lead to over- or underestimation of PNML. At present, there is no universally accepted population biomarker to account for population movement. This remains an active area of research, and the integration of multiple biomarkers could represent an important future direction which could refine population estimates and improve the robustness of cross-national WBE assessments.

4. Conclusions

The present study provides a snapshot of the international prevalence of NPS in influent wastewater from 52 sites across 20 countries during the 2022/2023 New Year. A total of 21 NPS were detected in this work. These findings were based on an updated analytical method, applied to the emerging compounds relevant at the time of study. The most commonly identified NPS were synthetic cathinones, benzodiazepines, and phencyclidine-type substances, with mitragynine, 3-MMC, and eutylone being the most frequently reported compounds. In addition to the spatiotemporal investigation of NPS occurrence, the study examined temporal trends of 3-MMC to understand the general declining trend of 3-MMC PNML mass loads. The decline in European cities could be associated with the enhancement of existing local legislation after Covid-19, in addition to ongoing scheduling of 3-MMC in the European Union and at the international level. Furthermore, the evaluation of the loaded SPE cartridge provides a body of knowledge on the stability of NPS compounds at various storage conditions, with −20 °C being the ideal temperature up to four months. As new NPS emerge onto the drug market, spatiotemporal wastewater analysis in combination with scheduling information of controlled NPS can offer insights into dynamic drug markets. The present work demonstrated the possible application of wastewater as an auxiliary assessment tool to understand the effectiveness and impact of scheduling of NPS through long-term surveillance.

Declaration of generative AI and AI-assisted technologies in the writing process

Declaration of generative AI and AI-assisted technologies in the writing process. During the preparation of this work the author(s) used ChatGPT-4o to check grammar, spelling, and improve the readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Dhayaalini Nadarajan: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Laimou-Geraniou:** Writing – review & editing, Resources, Formal analysis. **Andrew Chappell:** Resources, Formal analysis. **Christine Baduel:** Resources, Formal analysis. **Lubertus Bijlsma:** Writing – review & editing, Resources, Formal analysis. **Tim Boogaerts:** Writing – review & editing, Resources, Formal analysis. **Daniel A. Burgard:** Resources, Formal analysis. **Sara Castiglioni:** Writing – review & editing, Resources, Formal analysis. **Nicola Ceolotto:** Resources, Formal analysis. **Erin M. Driver:** Resources, Formal analysis. **Fernando Fabriz Sodre:** Writing – review & editing, Resources, Formal analysis. **Despo Fatta Kassinos:** Writing – review & editing, Resources, Formal analysis. **Harold Flores Quintana:** Writing – review & editing, Resources, Formal analysis. **Cobus Gerber:** Writing – review & editing, Resources, Formal analysis. **Emma Gracia-Lor:** Resources, Formal analysis. **Elisa Gracia-Marín:** Writing – review & editing, Resources, Formal analysis. **Rolf U. Halden:** Writing – review & editing, Methodology, Formal analysis. **Ester Heath:** Writing – review & editing, Resources, Formal analysis. **Julia Huchthausen:** Resources, Formal analysis. **Barbara Kasprzyk-Hordern:** Writing – review & editing, Resources, Formal analysis. **Emma L. Keller:** Writing – review & editing, Resources, Formal analysis. **Foon Yin Lai:** Writing – review & editing, Resources, Formal analysis. **Arndís Sue-Ching Löve:** Writing – review & editing, Resources, Formal analysis. **João Matias:** Writing – review & editing. **Vera Ocenaskova:** Resources, Formal analysis. **Jeong-Eun Oh:** Resources, Formal analysis. **Temilola Oluseyi:** Resources, Formal analysis. **Kaitlyn Phung:** Resources, Formal analysis. **Marco Pineda-Castro:** Resources, Formal analysis. **Magda Psichoudaki:** Writing – review & editing, Resources, Formal analysis. **Noelia Salgueiro-Gonzalez:** Writing – review & editing, Resources, Formal analysis. **Cezar Silvino-Gomes:** Writing – review & editing, Resources, Formal analysis. **Fábio Marley Sousa Nascimento:** Resources, Formal analysis. **Bikram Subedi:** Resources, Formal analysis. **Nikolaos Thomaidis:** Resources, Formal analysis. **Natan Van Wichelen:** Writing – review & editing, Resources, Formal analysis. **Degao Wang:** Resources, Formal analysis. **Viviane Yargeau:** Resources, Formal analysis. **Sarah Cresswell:** Writing – review & editing, Supervision. **Jochen F. Mueller:** Writing – review & editing, Visualization, Supervision, Resources. **Richard Bade:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Despo Fatta-Kassinos, an author of this submission, serves as an editor of Water Research. The manuscript was handled and reviewed independently by other editors, and Despo Fatta-Kassinos is recusing herself from any decisions related to the manuscript evaluation and acceptance. The other authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in

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Data availability

Data will be made available on request.

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