Received: 3 October 2024 | Accepted: 23 January 2025

DOI: 10.1111/add.70027

RESEARCH REPORT

ADDICTION SSA

Early identification of the use of potent benzylbenzimidazoles (nitazenes) through wastewater analysis: Two years of data from 22 countries

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Funding information
University of Queensland; Queensland Health; Australian Research Council, Grant/Award Number: DE220100381

Abstract

Background and Aims: The use of new synthetic opioids, such as the highly potent 2-benzylbenzimidazoles (i.e. nitazene) drugs, is a global health concern because of their increased risk of fatal overdose. In the early 2020s, nitazene analogues were linked to significant numbers of overdoses in the United States. Their reach is now worldwide, with nitazene overdose deaths reported in Europe, Australia and New Zealand. The aim of this study was to measure quantities of nitazenes in wastewater samples collected from 68 locations in 22 countries, covering six continents, to understand and estimate their use.

Methods: Untreated influent wastewater samples were collected over a one-week period that included the New Year period in 2022–2023 and 2023–2024. Samples were collected from 22 countries: Australia, Austria, Belgium, Brazil, Canada, Chile, China, Cyprus, Czechia, France, Germany, Greece, Iceland, Italy, New Zealand, Nigeria, Republic of Korea, Slovenia, Spain, Sweden, United Kingdom and United States. Samples were loaded onto solid-phase extraction cartridges in the country of collection and sent to Australia for elution and analysis using sensitive liquid chromatography–mass spectrometry methods.

Results: A total of 683 individual wastewater samples were analysed across the two years: 339 in 2022–2023 and 344 in 2023–2024. Two nitazene analogues—protonitazene and N-pyrrolidino etonitazene (etonitazepyne)—were found in five separate sites in the United States and Australia. In the 2022–2023 period, protonitazene was found in two sites in the United States. The following year, protonitazene was detected in two further sites in the United States, while both protonitazene and etonitazepyne were found in one site in Australia. Protonitazene mass loads ranged between 0.3 mg/day/1000 people and 100 mg/day/1000 people. Etonitazepyne was also found at mass loads between 0.2–2 mg/day/1000 people).

Conclusions: A very high mass load of protonitazene was calculated, using wastewater analysis, for the day of 30 December 2023 in one site in Australia. Etonitazepyne showed the same trend from a lower base. Wastewater-based nitazene surveillance shows promise as a form of both drug early warning and ongoing monitoring of trends in use, especially as a complementary tool to existing surveillance methods.

KEYWORDS
early warning system, etonitazepyne, illicit drugs, new psychoactive substances, protonitazene, wastewater-based epidemiology

INTRODUCTION

The new synthetic opioids (NSO) 2-benzylbenzimidazoles (nitazenes) are an increasing global health concern. These highly potent compounds first emerged on the illicit drug market in the late 2010s and are now one of the fastest growing groups of new psychoactive substances (NPS) in the world [1–3].

Nitazenes were developed in the late 1950s as an alternative to morphine, but never marketed because of concerns about their toxicity [4]. They consist of a benzimidazole core substituted with a 2-aminoethylside chain and a phenylalkyl chain [5, 6]. They are μ opioid receptor agonists with potency that ranges from that comparable to fentanyl (e.g. metonitazene), to 45-fold greater (N-desethyl isotonitazene), based on in vitro μ-opioid receptor activity assays [7].

Isotonitazene, the first to appear on the illicit drug market in 2019 after which it has come to dominate the NSO market, contributing to hundreds of fatalities in the United States (US) [8]. The dynamic nature of these compounds was shown in Tennessee, where there four times as many nitazene-related overdoses in 2021 as in the previous year, with the specific nitazene changing from isotonitazene to metonitazene [9]. NSOs have since rapidly expanded to global drug markets, driven by their ease of transportation, higher potency and lower costs for distributors and consumers [10].

Exposure to nitazenes is often unexpected. Many persons have been sold nitazene-based products that they believed to contain other drugs [11, 12]. In Australia, public health alerts have been issued in almost every jurisdiction about nitazene products that have been sold as methamphetamine and γ hydroxybutyrate (GHB) [13], 3,4-methyl enedioxy methamphetamine (MDMA), cannabinoid vapes and counterfeit benzodiazepines [14], heroin, cocaine and ketamine [15]. Unwitting use of nitazenes greatly increases the risks of toxicity and fatal overdose.

Since 2023, protonitazene and metonitazene have become the dominant NSOs used globally. A United Nations Office of Drugs and Crime report found these nitazenes were the most prevalent NSOs in clinical cases, fatal overdoses and driving under the influence cases from 12 Member States from the Americas, Europe, Asia and Oceania between December 2021 and May 2023 [16]. The recent emergence of ultrapotent nitazenes, the *N*-pyrrolidino analogues, has amplified public health concern because their potency have been reported to be up to 45 times that of fentanyl [7]. A looming synthetic ultrapotent opioid crisis has been described as a 'foreseeable disaster' [17]. However, a comprehensive picture on NPS is problematic because of the traditionally applied surveillance tools.

Wastewater-based epidemiology (WBE) is a monitoring modality that has been used for over a decade internationally to monitor the use of illicit drugs in the community [18]. New methods of wastewater analysis can detect concentrations as low as 0.01 ng/L. This sensitivity addresses challenges relating to the relatively unknown metabolism of NPS, and the low doses that are typically consumed. These methods provide an invaluable tool to monitor population-level use of NPS [19, 20]. The NPS Consortium have previously shown the utility of WBE to monitor spatial and temporal changes internationally [21].

A recent study detected protonitazene in wastewater for the first time in samples collected in Washington and Illinois, United States (US) between 27 December 2022 and 4 January 2023 [22]. This was the first to do so globally. However, wastewater drug monitoring programs do not routinely test for these compounds. We, therefore, explored WBE to estimate global use of nitazenes in 68 locations, 22 countries capturing more than 27 million people, in 2 week-long wastewater sampling campaigns conducted over the New Year period of 2022 to 2023 and 2023 to 2024. These sampling dates were chosen because of expected increase in drug use over this period. With nitazenes having been found as an adulterant in a range of drugs including stimulants, cannabinoids, opioids and benzodiazepines, it was thought that this time period would provide a higher chance of capturing any nitazene consumption.

METHODS

Untreated influent wastewater samples (200–500 mL) were collected for at least 3 days over the New Year period from 68 locations in 22 countries. Samples from all but one site were collected over a 7-day period with New Year's Eve in the middle (i.e. 28 December 2022–4 January 2023 and 28 December 2023–4 January 2024). One site in the United Kingdom collected five individual samples over a 2-week period leading up to Christmas 2022. Individual sampling dates are presented in Data S1.

There were 51 locations in 20 countries (2022/23) and 58 locations in 22 countries (2023/2024), respectively, covering a global population of more than 27 million inhabitants (Data S1), with 41 sites collecting samples in both years. The participating sites were from an ongoing international wastewater surveillance project (NPS Wastewater Consortium), with new sites added yearly. Samples were collected from Australia (up to 10 sites per year), Austria (1), Belgium (2), Brazil (5), Canada (1), China (1), Chile (3), Cyprus (2), Czechia (2), Germany (2), Spain (3), France (1), Greece (1), Iceland (1), Italy (1), Republic of Korea (1), Nigeria (1), New Zealand (4), Sweden (1), Slovenia (7), United Kingdom (2) and United States (10), shown graphically in Figure 1.

All collaborators followed a common protocol for wastewater sampling and extraction. They were instructed to collect 24-hour composite samples using flow- or time-proportional autosamplers. The samples were then acidified to pH 2 using hydrochloric acid on collection and stored frozen until all samples had been collected. At the conclusion of the sampling period, the samples were transported

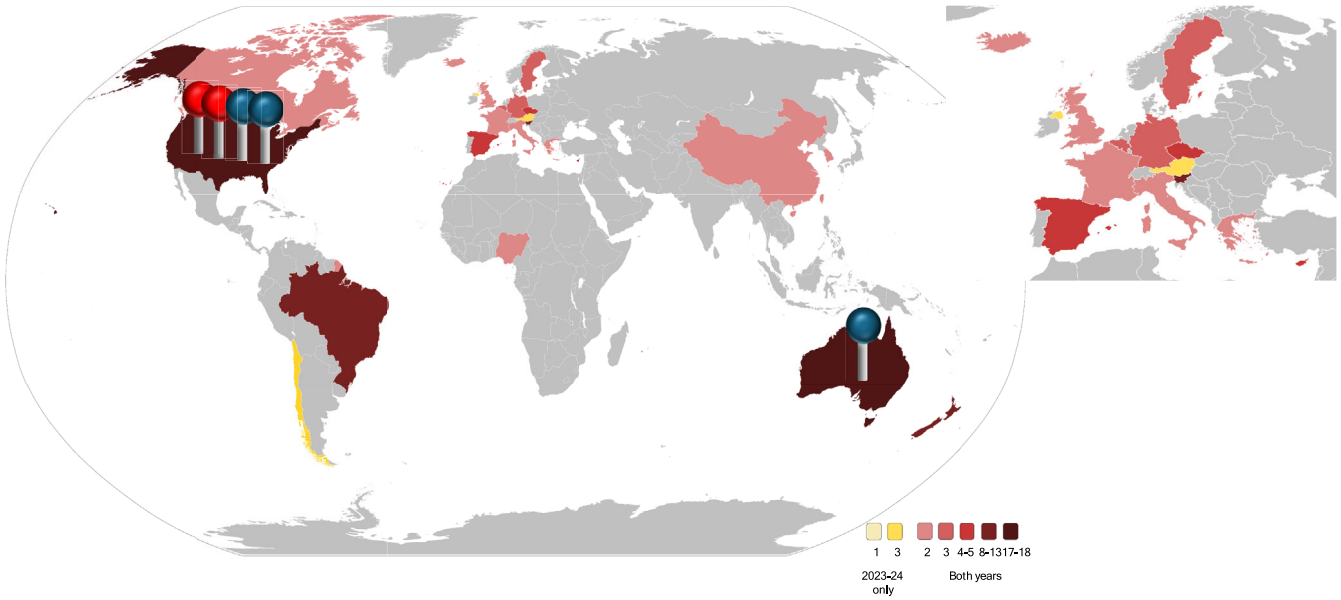

FIGURE 1 Map of sites included in this work. Number of sites is across both years, with sites only included in the most recent campaign in shades of yellow. Europe is shown as an insert to better portray coverage. Individual sites and associated populations are included in the supporting information. Pins show sites within countries where nitazenes were found (red = 2022/2023; blue = 2023/2024).

to a laboratory in the home country and stored frozen at −20°C until sample processing. To allow for semi-quantitative analysis, deuterated internal standards of illicit drugs from each collaborators' laboratory were spiked into the samples, as reported in previous work [18]. Internal standards were not available for samples collected from six sites (Data S1), in which case results were only qualitative.

Samples were processed using a previously validated solid-phase extraction (SPE) method, using UCT XtracT DAU cartridges (UCT, Bristol, PA) [23]. Samples (100 mL) were loaded on to the cartridges, dried, and then sent to The University of Queensland, Australia for elution and analysis. The international shipment of dried SPE cartridges has been successfully undertaken in several previous studies, including our work on the first detection of protonitazene in wastewater [21, 22, 24]. We have specifically investigated the on-cartridge stability of protonitazene as a proxy for nitazene-analogues, and it was found to be stable for at least 14 days at temperatures up to room temperature (22°C), covering the shipment time from all locations to Australia.

All samples were analysed on a previously validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, with specific information included in Data S1. Eight NSOs were included in this work: butonitazene, etodesnitazene, etonitazepyne, flunitazene, isotonotazene, metonitazene, N-desethyl isotonitazene and protonitazene. Additional information about sample analysis, including quality controls, quality assurance and calculations are also provided in Data S1. Samples in which nitazenes had been detected following the LC–MS/MS protocol were also reanalysed using a LC-high resolution MS suspect screening workflow to investigate the presence of metabolites. Specific details are outlined in Data S1.

Concentrations were transformed to population normalised mass loads by incorporating flow rates (megalitres) and population (in 1000s), provided by the collaborators.

The analysis in this study was not pre-registered, and as a result, the findings should be considered exploratory.

RESULTS

A total of 683 individual wastewater samples were analysed across the 2 years, 339 in 2022 to 2023 and 344 in 2023 to 2024. The method limit of detection (LOD) was deemed to be 0.2 ng/L, whereas the limit of quantification (LOQ) was 0.5 ng/L for both protonitazene and etonitazepyne. In the 2022 to 2023 collection, protonitazene was found above the limit of detection in two samples from two sites in the United States, at concentrations of up to 0.5 ng/L, and estimated mass load of up to 0.3 mg/day/1000 people, as previously published [22]. In the 2023 to 2024 collection, protonitazene was found above the limit of detection in two sites (across four individual samples) in the United States and one in Australia. The samples in the United States were at levels below the LOQ, but above the LOD for our method. The site in Australia had levels of between 5 and 300 ng/l across the sampling week, at an estimated mass load of between 2 and 100 mg/day/1000 people. *N*-Pyrrolidino etonitazene (etonitazepyne) was also found in the same site in Australia, at concentrations between 0.5 and 5 ng/L (0.2–2 mg/day/1000 people). Daily trends are shown in Figure 2.

DISCUSSION

Our findings are supported by previous literature, which has found protonitazene to be the predominant NSO globally [25]. Indeed, previous published case reports of protonitazene in wastewater derive

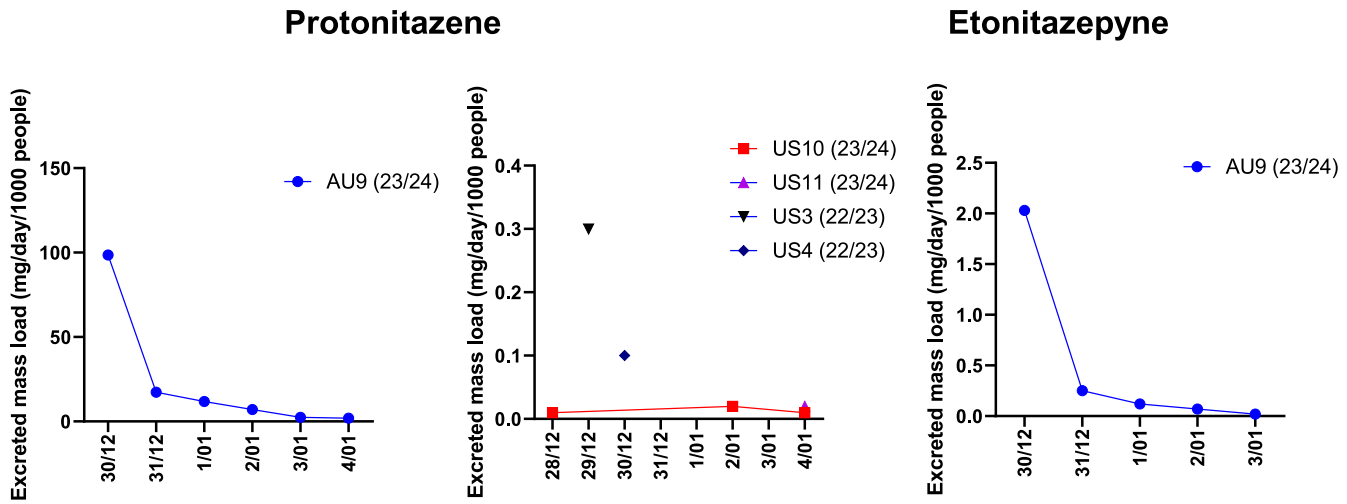

FIGURE 2 Estimated mass loads of the nitazene analogues found in this study. Site-specific information is found in Data S1. Note: for graphical purposes, the levels in the US sites detected below the limit of quantification (i.e. US10, US11 and US4) were determined as the midpoint of the limit of detection and limit of quantification. AU, Australia; US, United States.

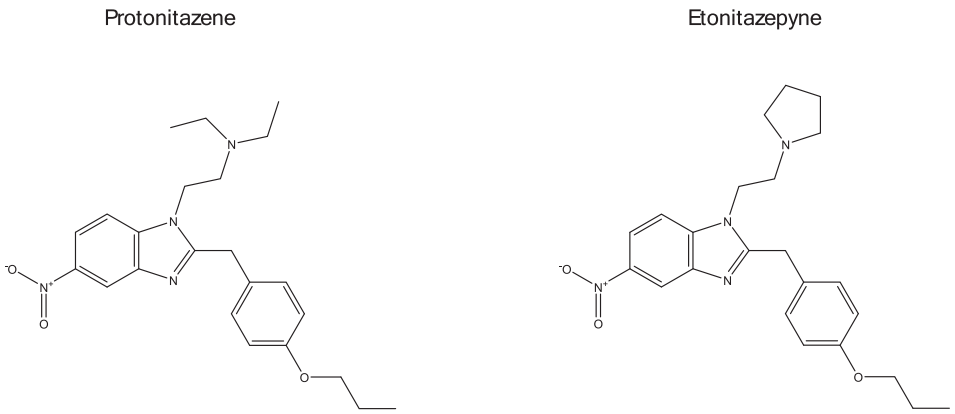

FIGURE 3 Structures of protonitazene and N-Pyrrolidino etonitazene (etonitazepyne).

from North America [22], where we detected this compound in four separate sites across the two sampling periods. Protonitazene was first detected in Canada and the United States in 2020 and in Australia in 2022 [26]. It has an efficacy of approximately 1.07 to 1.29 times greater than fentanyl and more than 130 times greater than morphine. This explains the low blood concentrations detected in overdose. In 45 post-mortem cases reported to the UNODC Early Warning Advisory, the median protonitazene blood concentration was 1.6 (range, 0.57–17.0) ng/mL [27].

N-Pyrrolidino etonitazene (etonitazepyne) differs structurally from other nitazenes [28] in a way that makes etonitazepyne approximately 40 times more potent than fentanyl [7], with greater activation of the β-arrestin2 pathway (EC50 = 0.548 nM). The structures of both protonitazene and etonitazepyne are shown in Figure 3. The latter effect produces more pronounced respiratory depression than traditional opiates [1]. Etonitazepyne was first reported in Europe in February 2021 [28]. It reached New Zealand and the United States by May 2021, and there were reports of counterfeit oxycodone tablets containing the compound in Wales [29].

This compound was detected in US wastewater during the study period, but *post-mortem* data reveals that it was present in the eastern states as early as March 2021 [30]. A public health alert was released in the United States after eight deaths between January and April 2021 [31] in decedents between 20 and 60-years-old [30]. Etonitazepyne was detected in blood at 8.3 ng/mL in one case with no other significant co-exposures and 2.4 ng/mL in another case where other drugs were detected. In October 2022, 21 deaths were reported across North America, most of which were co-detected with other drugs, including fentanyl (n = 12), methylamphetamine (n = 12) or benzodiazepines (n = 11) [1].

We did not detect any nitazenes in wastewater samples collected from the United Kingdom, despite growing reports of nitazenes in this region over the past year [32]. However, as wastewater analysis is focussing on a community rather than individuals, small nitazene outbreaks within a large wastewater treatment plant catchment area could be difficult to detect in the wastewater because of dilution effects. In addition to increasing fatalities, there are reports of non-fatal overdoses across the United Kingdom, leading to concerns of a spiralling NSO crisis, particularly in response to recent changes in

heroin availability in the region [32]. This concern has been echoed by the European Union Drugs Agency, with data from the Baltic regions demonstrating increasing nitazene-related mortality between 2022 and 2023 [33]. Nitazene detections in Estonia increased from 39% to nearly half (48%) of all drug-related deaths during this period, whereas in Latvia the increase was more significant, which went from 3% to 29%. Deaths were also reported in France and Ireland, reinforcing the threat of nitazenes across the continent [33]. Previous wastewater analysis for nitazenes has been undertaken in Europe, but none have yet been detected [34].

This recent shift in the drug market has also been reported in São Paulo, Brazil, where nitazenes were detected in 95% of total opioid related state seizures between July 2022 and April 2023 [35]. Interestingly, they were typically found in herbal matrices for smoking and were mixed with synthetic cannabinoids, MDMB-4en-PINACA and ADB-BUTINACA were the most common, reflecting formulations specific to the South American region. It must be mentioned that the current work did not include samples from São Paulo, with the high detection frequency of nitazenes in opioid seizures potentially a regional trend.

Consumption versus disposal

Because of the large discrepancy in protonitazene mass loads between the site in Australia and the sites in the United States, an additional investigation assessed whether this could have been because of direct disposal. Several previous studies have differentiated direct disposal from consumption by using the ratios between metabolites and parent compounds, enantiomeric profiling, specific metabolites as well as specific weekly trends [36–39].

In the current study, a very high mass load of protonitazene was calculated for the day of 30 December 2023, with nothing detected the previous day (Figure 2). The mass load decreased significantly the following day, with a gradual decrease seen until sampling ended on 4 January 2024. Etonitazepyne showed the same trend, albeit from a lower base. Reliable, comprehensive pharmacokinetic data were unavailable to back-calculate the excreted mass loads in the work to estimate consumed loads, but with such high levels on 30 December, the samples were reanalysed using suspect screening (Data S1) incorporating published metabolites of protonitazene, such as including N-desethyl-protonitazene, 5-amino-protonitazene and 4-hydroxy-nitazene, which have been identified in human urine [40]. 4-Hydroxy nitazene has previously been hypothesised as a universal metabolite of nitazene analogues [8, 40]. Reference standards of these metabolites were not available in our laboratory at the time of the study. However, after the suspect screening analysis, none of these metabolites were detected in any sample, indicating potential for direct disposal. However, further investigation is needed to determine in-sample stability of these metabolites to completely rule out consumption.

These detections were reported to the public health agency in the relevant jurisdiction, which evaluated them against their active and passive drug surveillance system including opioid-related ambulance attendances, opioid-related emergency department presentations, opioid-related deaths, nitazene detections in police seizures, emergency department toxicology and post-mortem toxicology. No corresponding signals were identified. Therefore, both wastewater and public health data sources suggested that the protonitazene and etonitazepyne detected in Australia were the result of direct disposal, either as a mixture or individually. In the months that followed this finding, protonitazpyne and protonitazene were deemed to be the cause of severe opioid overdoses in this same jurisdiction. The detection of these nitazenes in wastewater led to heightened toxicovigilance by health services in this jurisdiction.

Potential of wastewater analysis for surveillance of nitazenes

The primary strength of wastewater analysis for the surveillance of nitazenes is as a complementary data source. Nitazene analogues are rapidly emerging around the world and because of their potency and potential for unwitting consumption in a range of illicit drugs, it is important from a public health perspective to have as many data sources on their use as possible to reduce harm. For example, several recommendations have been suggested to make more accurate and timely identification of nitazene-related deaths in Australia, including the development of early reporting systems from multiple data sources such as coroners, forensic toxicology labs and drug surveillance programs [41]. Wastewater can provide an insight into community trends rather than individuals and so can objectively analyse their use in larger populations.

The current wastewater analysis has limitations. It is important to note that although the current work is the most comprehensive international wastewater surveillance of nitazenes, one site does not necessarily reflect widespread use across the country. Conversely, the non-detections of nitazenes in our study sites does not mean that there was no nitazene use in that location. Moreover, the high potency of nitazenes means that the expected dose size is very low compared to stimulants (i.e. MDMA; 100 mg) or even fentanyl (0.2 mg). Combined with potentially a small user pool and the dilution of a wastewater treatment plant catchment area, nitazenes may be difficult to detect despite a highly sensitive analytical method.

A limitation common to all surveillance methods for NPS is that they comprise a dynamic unregulated illicit drug market, in which new analogues continually emerge. Although the nitazenes included in our method were the most prevalent at the time of the study, newer analogues have emerged in the more recent months.

Wastewater analysis has been embraced by international agencies including the United Nations Office on Drugs and Crime [42], European Union Drugs Agency [43], as well as national programs established in Australia [44] and New Zealand [45], as a means to understand the community use of illicit drugs including MDMA, cocaine, heroin, methamphetamine and cannabis. The current work

has shown the potential of wastewater analysis to build on these campaigns and serve as a complementary early warning device for nitazenes to better inform public health authorities about their use.

International Collaboration

In this work, we collected and analysed wastewater from 22 countries across six continents to provide a snapshot of nitazene activity during the New Year period of 2022 to 2023 and 2023 to 2024. We have demonstrated that nitazene analogues can be detected in wastewater, using a highly sensitive method.

Many countries used wastewater surveillance during the coronavirus disease 2019 pandemic to understand the dynamics around viral mutations and to determine which communities had been impacted. The current work shows that these frameworks can be harnessed to also monitor chemicals, including different classes of illegal drugs such as these NSOs. Moreover, findings from wastewater data can be disseminated to local, national and international agencies to ensure that harms are reduced from nitazene consumption. For example, illicit drug use is currently monitored through wastewater analysis in New Zealand, Australia and across Europe, with the programs supported by both criminal (i.e. Australian Criminal Intelligence Commission, New Zealand Police) and health (European Union Drug Agency) agencies. Incorporating nitazene analysis into these ongoing monitoring regimes has the potential to identify emerging analogues at a population level so that timely public health responses and targeted policies can reduce the risks of harm.

CONCLUSION

Nitazenes continue to pose a serious threat to public health, particularly because of their high potency and contamination of non-opioid drugs such as benzodiazepines, ketamine and cocaine. There is an elevated overdose risk compared to other drugs such as fentanyl, which has driven the overdose crisis in North America over the last decade. WBE has established itself as an important tool for monitoring illicit drug use in the population and is a promising complementary tool for monitoring nitazenes and providing an early warning system.

Despite their low dose sizes, and therefore, low levels expected, our work has shown that they can be detected in wastewater. Analyses can be conducted in real-time, and data obtained within days-weeks so that findings could be relayed to relevant public health authorities. The incorporation of nitazenes into ongoing wastewater monitoring programs can provide authorities with earlier identification of emerging analogues and increased use of these compounds, enabling rapid implementation of public health interventions before the harms occur and before they become more widespread.

AUTHOR CONTRIBUTIONS

Richard Bade: Conceptualisation (lead), writing—original draft (equal), visualisation (lead), data curation (equal), supervision (lead). **Dhayaalini Nadarajan**: Investigation (lead), data curation (equal), formal analysis (lead), methodology (equal), validation (equal). **Wayne Hall**: Writing—review and editing (equal). **Jared Brown**: Investigation (equal), writing—review and editing (equal). **Jennifer Schumann**: Writing—original draft (equal), conceptualisation (supporting).

ACKNOWLEDGEMENTS

We sincerely thank the wastewater treatment plant operators for their help in collecting samples and the support provided by the lab staff who performed the extraction of the samples. We also thank SCIEX for the use of their instrument. The Queensland Alliance for Environmental Health Sciences, The University of Queensland, gratefully acknowledges the financial support of Queensland Health, Australia. The National Centre for Youth Substance Use Research receives funding from the Commonwealth Department of Health. R. B. is the recipient of an Australian Research Council Discovery Early Career Award (project number DE220100381) funded by the Australian Government. The views expressed in this article are those of the author(s) and not necessarily those of the funding agencies. Open access publishing facilitated by The University of Queensland, as part of the Wiley - The University of Queensland agreement via the Council of Australian University Librarians.

DECLARATIONS OF INTEREST

None.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Bade R, Nadarajan D, Hall W, Brown JA, NPS Wastewater Consortium, Schumann J. Early identification of the use of potent benzylbenzimidazoles (nitazenes) through wastewater analysis: Two years of data from 22 countries. Addiction. 2025. https://doi.org/10.1111/add.70027