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Research Paper

Wastewater-based epidemiology for comprehensive community health diagnostics in a national surveillance study: Mining biochemical markers in wastewater

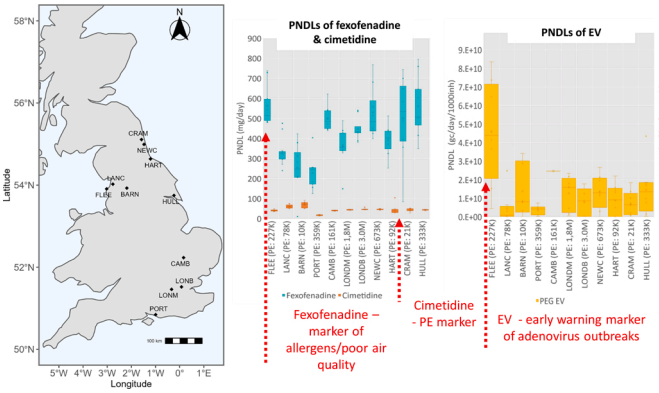
Barbara Kasprzyk-Hordern [a,*], Natalie Sims [a], Kata Farkas [b], Kishore Jagadeesan [a], Kathryn Proctor [a], Matthew J. Wade [d], Davey L. Jones [b,c]

[a] Department of Chemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK
[b] Centre for Environmental Biotechnology, School of Natural Sciences, Bangor University, Bangor, Gwynedd LL57 2UW, UK
[c] Food Futures Institute, Murdoch University, Murdoch WA 6105, Australia
[d] Analytics & Data Science Directorate, UK Health Security Agency, London SW1P 3JR, UK

HIGHLIGHTS

- First national WBE study encompassing both chemical and biological markers.
- Community-wide chemical intake informs disease status and hazardous chemical exposure.
- Direct disposal of unused pharma and industrial discharge plasticisers observed.
- Endogenous health markers important as generic markers of health status in communities.
- Virus markers are highly variable with clear cases of localized outbreaks observed.

GRAPHICAL ABSTRACT



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ABSTRACT

This manuscript showcases results from a large scale and comprehensive wastewater-based epidemiology (WBE) study focussed on multi-biomarker suite analysis of both chemical and biological determinants in 10 cities and towns across England equating to a population of ~7 million people. Multi-biomarker suite analysis, describing city metabolism, can provide a holistic understanding to encompass all of human, and human-derived, activities of a city in a single model: from lifestyle choices (e.g. caffeine intake, nicotine) through to health status (e.g. prevalence of pathogenic organisms, usage of pharmaceuticals as proxy for non-communicable disease, NCD, conditions or infectious disease status), and exposure to harmful chemicals due to environmental and industrial sources (e.g. pesticide intake via contaminated food and industrial exposure). Population normalised daily loads (PNDLs) of many chemical markers were found, to a large extent, driven by the size of population contributing to wastewater (especially NCDs). However, there are several exceptions providing insights into chemical intake that

* Corresponding author.
E-mail address: B.Kasprzyk-Hordern@bath.ac.uk (B. Kasprzyk-Hordern).

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can inform either disease status in various communities or unintentional exposure to hazardous chemicals: e.g. very high PNDLs of ibuprofen in Hull resulting from its direct disposal (confirmed by ibuprofen/2-hydroxyibuprofen ratios) and bisphenol A (BPA) in Hull, Lancaster and Portsmouth likely related to industrial discharge. An importance for tracking endogenous health markers such as 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA, an oxidative stress marker) as a generic marker of health status in communities was observed due to increased levels of HNE-MA seen at Barnoldswick wastewater treatment plant that coincided with higher-than-average paracetamol usage and SARS-CoV-2 prevalence in this community. PNDLs of virus markers were found to be highly variable. Being very prevalent in communities nationwide during sampling, SARS-CoV-2 presence in wastewater was to a large extent community driven. The same applies to the fecal marker virus, crAssphage, which is very prevalent in urban communities. In contrast, norovirus and enterovirus showed much higher variability in prevalence across all sites investigated, with clear cases of localized outbreaks in some cities while maintaining low prevalence in other locations. In conclusion, this study clearly demonstrates the potential for WBE to provide an integrated assessment of community health which can help target and validate policy interventions aimed at improving public health and wellbeing.

1. Introduction

In 2020 we witnessed an unprecedented crisis in healthcare systems due to the COVID-19 pandemic that has highlighted the importance of rapid population-wide surveillance tools for fast recognition and response to changes in infections/prevalence. Wastewater-based epidemiology (WBE) has become a complimentary tool that has enabled effective SARS-CoV-2 surveillance in the UK [21,25,53,20] and globally [7]. WBE is currently used to estimate illicit drug use at both national and international scales [41,51,23,28]. Further, WBE has also been utilised to estimate usage of other lifestyle chemicals such as alcohol [43,5], tobacco [8], new psychoactive substances [9], counterfeit medicines [52,11,12], sweeteners [35], antibiotics and corresponding resistance genes [10,14] as well as levels of stress biomarkers such as isoprostanes [40,47], or histamine burden [13]. WBE has revolutionised population health studies, especially in the context of the COVID-19 pandemic [3,38,49,7]. WBE has also focussed on public exposure to chemicals such as pesticides [45,40,46] and industrial chemicals [37,36,6,22,39,50] as well as pharmaceuticals [1,2,32,33,44,54,31,6]. Unfortunately, there has been little progress in linking biological determinants with chemical markers to provide a truly integrated and comprehensive evaluation of public health.

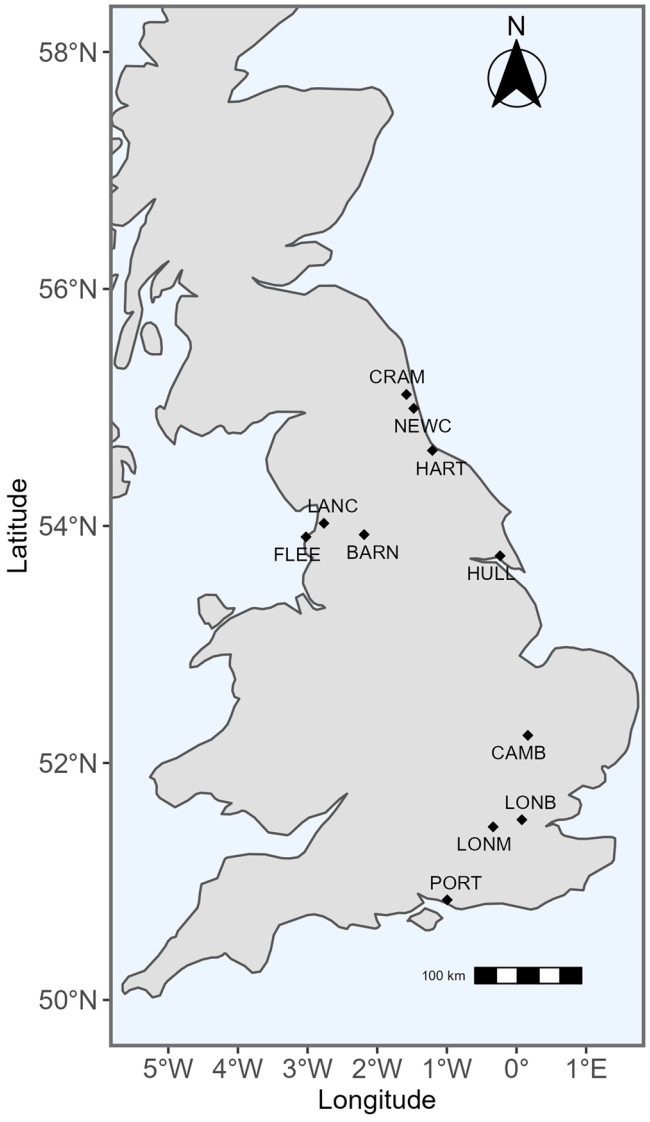

Fig. 1. Study sites (WWTPs), biochemical indicators (BCIs) and population equivalents (estimates using General Practice (GP) healthcare facilities (PE-NHS) and data provided by the water companies (PE-WW)).

Due to the wealth of data that can be generated from wastewater monitoring, there is also a need to embrace the advancement of big data and mass spectrometry driven WBE systems for new biological and chemical threats. These developments will require the integration of bioanalytical tools to inform decision-making regarding site selection, subsequent sampling and the building of early warning systems to rapidly inform public policy. To contribute to the development of an evidence base supporting this opinion, we describe a proof-of-concept project delivering a national WBE surveillance study in a close collaboration of WBE chemical analysis experts, WBE virologists and government agencies (UK Health Security Agency and Environment Agency).

This paper presents a novel integrated approach towards a broad-biomarker spectrum data collection aimed at comprehensive wastewater profiling encompassing both chemical and biological targets. Several biomarker groups were selected including viral targets (SARS-CoV-2, norovirus and adenovirus), chemical markers (pharmaceuticals used in infectious disease and non-communicable disease (NCD) treatment, industrial chemicals, endogenously formed chemicals markers) and traditional water quality indicators (WQI). Such an approach is fundamentally important to develop WBE as a key One Health support tool.

2. Experimental

2.1. Biochemical Indicators (BCIs)

Several BioChemical Indicator (BCI) groups were targeted in this study (Fig. 1). These are: viral targets (SARS-CoV-2, norovirus and adenovirus) and chemical targets: pharmaceuticals, metabolites, lifestyle chemicals, industrial and personal care products. Traditional water quality indicators (WQI) (ammonium, orthophosphate, pH, electrical conductivity and turbidity) were also included. These BCIs were selected

to provide a national study coverage with a wide spectrum of analytes.

2.2. Selected WWTPs sites and sampling

Samples were collected on nine randomly selected days (4–5 days every week, including weekends) in November 2021 using 24 h composite sampling (with subsamples collected at 15 min and 1 h intervals using refrigerated autosamplers where available). Two out of 11 sites had grab samples provided on occasion: Lancaster (Stodday WWTP): 10 out of 10 samples and Portsmouth & Havant WWTP: 3 out of 10 samples.

All collected samples were separated into two sample sets. One set of 500 mL PET bottles were filled with wastewater and transported to Bath on ice at least four times a week for two weeks (12–25 November 2021). On arrival (on average within <24 h after collection) samples were inspected to ensure they remained frozen during transport. They were then transferred to a freezer before processing (usually within a further 24 h window) (Fig. S1). A second set of identical bottles was transported refrigerated within 24 h after collection directly to Bangor for viral analysis.

Eleven wastewater treatment plants (WWTPs) were selected (Fig. 1) based on the following criteria: (i) 24 h composite sampling (primary requirement), (ii) flow measurement on site (primary requirement), (iii) known WWTP population equivalent (PE) (primary requirement), (iv) WWTPs covering a wide PE range from 10^3 to $> 10^6$, (v) low industrial input (secondary requirement), (vi) very good geographical spread of WWTPs accounting for North, South, East and West England (vii) minimum of eight 24 h composite samples/WWTP, including sample collection during weekends.

2.3. Sample preparation and analysis of chemical markers with mass spectrometry-based methods

University of Bath Environmental Chemistry and Public Health Research Group workflows were utilised in this study (see Fig S1). All analytes and internal standards were of high purity (>97%). Water was purified using a Milli-Q purification system from Millipore (Nottingham, UK). Methanol, formic acid (>95%), NH_4OH, NH_4F were purchased from Sigma (UK) and Fisher (UK). All solvents used were of HPLC grade or higher.

2.3.1. Solid-Phase Extraction (SPE)

Wastewater samples were portioned into 50 mL aliquots before measuring the pH and adjusting to 7.5 ± 0.5 if required. Samples were then spiked with IS (Internal Standard) mix (100 ng/compound) (Table S1) before filtering through glass microfibre GF/F (0.7 μm) filters. Analytes of interest were extracted from the filtrate using solid phase extraction (SPE). This was achieved by loading the filtrate under vacuum onto pre-conditioned Oasis HLB (Hydrophilic Lipophilic Balance) cartridges (2 mL MeOH followed by 2 mL H_2O) at approximately 5 mL min^{-1}. Elution was carried out using 4 mL of MeOH. The eluent was collected in deactivated glass vials (Fisher, UK). The extracted samples were then dried under nitrogen at 40 °C, and resuspended in 500 μL of 80:20 H_2O:MeOH into liquid chromatography vials. Two full biological replicates were undertaken for every sample analysed.

2.3.2. NCD-UHPLC-QQQ

Analysis of chemical markers was achieved using an ACQUITY UPLC™ XEVO TQD-ESI triple quadrupole mass spectrometer (Waters, UK) coupled to an ultra-performance liquid chromatography instrument with an electrospray ionisation source (ESI). A reversed-phase BEH C18 column (150 ×1.0 mm, 1.7 μm particle size) was used for compounds' separation. The mobile phase rate was set at 0.04 mL min^{-1} with an injection volume of 15 μL. Two NCD markers methods have been selected, covering a broad range of analytes of interest, ESI + (34 min long) and ESI – (23 min long). Mobile phase conditions were as follows:

ESI + : Mobile phase A: H_2O:MeOH 95:5 + 0.1% formic acid, mobile phase B: 100% MeOH.

ESI –: Mobile phase A: H_2O:MeOH 80:20 + 1 mM NH_4F, mobile phase B: 5:95 H_2O: MeOH + 1 mM NH_4F.

For both methods, the source temperature was 150 °C and the desolvation temperature was 400 °C. The cone gas flow was 100 L/h and the desolvation gas flow was 550 L/h. Nitrogen was used as the nebulising and desolvation gas, and argon as the collision gas.

The coordinating program used was MassLynx V4.1, and the data processing program was TargetLynx (Waters Lab Informatics, UK). Validation parameters were determined per analyte to aid peak identification, including signal to noise (≥ 3 for detection, ≥ 10 for quantification); compound specific sensitivity limits (instrumental detection limit - IDL and instrumental quantification limit - IQL); and to be within stated thresholds for relative retention time and target ion ratio. All data points were accompanied by a quantitative pass/fail assessment for each parameter. MRM transition used as well as method performance criteria are gathered in Table S2-3.

2.3.3. AA-UHPLC-QQQ

Analysis of antimicrobial agents (AA) was achieved by using liquid chromatography-mass spectrometry (LC-MS/MS) and was performed using a Waters, ACQUITY UPLC™ system coupled to a Xevo TQD-ESI Mass Spectrometer and using a reverse-phase BEH C18 column (50 × 2.1 mm, 1.7 μm) with Acquity column in-line 0.2 μm pre-filter (Waters, UK). Conditions were optimised for fast chromatographic separation and high sensitivity across a range of drug classes. Chromatographic separation of each 20 μL sample was achieved over a 19 min gradient elution using H_2O:MeOH (95:5, with 0.1% formic acid), and methanol (100%) as mobile phases at a mobile phase flow of 0.2 mL min^{-1}.

The coordinating program used was MassLynx V4.1, and the data processing programs were QuanLynx and TargetLynx (Waters, UK). The integration parameters in QuanLynx (smoothing, apex track, and window extent) were optimised per analyte to minimise the effects of analyst subjectivity during data processing. Full method performance criteria can be found in [26].

2.3.4. Calculations

Daily mass loads of chemical markers, CIs (mg day^{-1}) were calculated by multiplying total CI concentrations (mg L^{-1}) in a 24 h composite raw wastewater sample by daily wastewater flow rates (L day^{-1}). Total CI concentrations in raw wastewater were calculated after accounting for both liquid and SPM (Suspended Particulate Matter) fractions using Eq. 1:

$$DL_{CI} = C_{CI} x V \quad (1)$$

where: C_{CI} is the total concentration of *CI* (mg L^{-1}) in influent wastewater (both liquid and SPE phase), *V* is the volume of wastewater received by the WWTP per day (L day^{-1}).

Population normalised daily mass loads of CIs (mg day^{-1} $1000inh^{-1}$) were calculated using Eq. 2:

$$PNDL_{CI} = \frac{DL_{CI}}{PE_{\phi}} x 1000 \quad (2)$$

where: DL_{CI} is the daily mass load of *CI* (mg day^{-1}) in influent wastewater, PE_{ϕ} is the population estimate of ϕ which refers either to the water utility PE estimate (PE_{WW}) or a population size of patients registered in primary care (PE_{NHS}), inh indicates inhabitants.

Statistical analysis was undertaken using Excel and Linear Regression Analysis. Constant values for population equivalents were applied for system calibration. Two population size estimates were used (Fig. 1): PE-WW and PE-NHS. PE-WWs were provided by water utilities. PE-NHS (population size by GP surgeries) were calculated based on the number of people registered in the GP surgeries located in the WWTPs catchment zone. GP surgeries information, such as, postcode and number of people registered were obtained from NHS Digital (https://digital.nhs.uk/). We

have used our in-house PrAna tool [30] to identify the GP surgeries present in each WWTPs catchment zone. The WWTPs catchment maps were used (with permission from all participating water utilities) to identify and collect GP surgeries information inside each catchment region, including number of patients registered using R for statistical computing and graphics. Further information on PE-NHS calculation can be found in Supplementary Information (SI).

2.4. Analysis of viral targets

Viral targets were analysed at Bangor University using two extraction methods: Polyethylene Glycol precipitation (PEG method) and Amicon® filtration (AM method) as described in [19] ([19]).

2.4.1. Modified Polyethylene Glycol (PEG) precipitation (BE-PEG method)

This method implemented a beef extract elution to detach viruses from solid matter prior to PEG precipitation [16]. The 200 mL samples and the same aliquot of water (serving as a control) were mixed with Lab Lemco beef extract (Oxoid, USA) and sodium nitrate to reach the final concentration of 3% and 2 M, respectively. The pH of the mixture was adjusted to 5.5 and then incubated at 50 rpm at room temperature for 30 min. The samples were centrifuged at 10,000 × *g* at 4 °C for 10 min to clarify the solutions. The pH of a 150 mL aliquot of the supernatants was adjusted to 7.0–7.5, spiked with the enveloped Phi6 process control virus and then were mixed with PEG 8000 and NaCl to reach the final concentration of 10% and 2%, respectively. The solutions were mixed by inverting several times and incubated at 4 °C for 16 h. The mixture was then centrifuged at 10,000 × *g* at 4 °C for 30 min. The resulting pellet was subject to RNA/DNA extraction.

2.4.2. Ultrafiltration using the Amicon® Ultra Centrifugal filters (Amicon method)

This method was adapted from [21,20] with small modifications. The samples (20 mL each) and a same aliquot of water (served as a control) were spiked with Phi6 virus and centrifuged at 4000 × *g* at 4 °C for 10 min to clarify the solutions. The pellets were discarded whereas 15 mL of the supernatants were transferred to 10 kDa Amicon® Ultra-15 centrifugal filters (Merck Life Science UK Ltd, Watford, UK). The samples were centrifuged at 5000 × *g* for 30–60 min to reach a final volume of 200–500 µL. The filtrates were discarded, and the concentrates were subject to viral RNA/DNA extraction.

2.4.3. Viral RNA/DNA extraction

Viral RNA/DNA of both concentrated pellets and unconcentrated samples were extracted using the NucliSens extraction system (BioMerieux, France) on a Kingfisher 96 Flex system (Thermo Scientific, USA) as described previously [18,34]. The final volume of the eluent was 100 µL.

2.4.4. Viral RNA/DNA quantification

The viral RNA/DNA were quantified with RT-qPCR (RNA targets) and with qPCR (DNA targets). All qPCR reactions were performed using a QuantStudio Flex 6 real-time PCR machine (Applied Biosystems Inc., USA). The primers and probes for the target viruses have been used and validated previously [18,34]. For quantification, a dilution series of DNA/RNA standards incorporating the target sequence were used. Each reaction plate contained multiple non-template controls, which were negative throughout the study, suggesting no cross-contamination.

The duplex and singleplex assays targeting RNA viruses used the TaqMan Viral 1-step RT-qPCR master mix (Applied Biosystems Inc., USA) with 1 µg bovine serum albumin (BSA) in the reaction mixes. Additionally, 16 nmol $MgSO_4$ was added to the reaction mix for the SARS-CoV-2, Phi6 and influenza targets. Duplex RT-qPCR assays were used for the SARS-CoV-2 N1 gene fragment and the Phi6 phage [34], for influenza A and B [48], for RSV A and B [24] and for enteroviruses (Public Health Wales, personal communication) and enterovirus D68 [42]. Singleplex assays were used for measles virus [29], for norovirus GI and GII [15], for rotavirus (RoV) (Primerdesign, UK). For human immunodeficiency virus (HIV), hepatitis B and C viruses we used a commercial triplex assay following the manufacturer's protocols

Table 1
Biochemical indicator daily loads in wastewater influent versus population size (calculated using PE-WW).

		AVERAGED (ONLY SPATIAL)			FULL DATASET (SPATIAL & TEMPORAL)		
Group	Compound	R^2	r	p-value	R^2	r	p-value
Analgesics and their metabolites	Tramadol	0.907	0.952	6.10E-02	0.590	0.768	1.82E-20
	N-demethylpregabalin	0.968	0.984	4.74E-08	0.902	0.950	9.58E-51
	Pregabalin	0.977	0.999	4.74E-08	0.822	0.950	9.58E-51
	Acetaminophen	0.906	0.952	6.61-06	0.788	0.888	2.03E-34
	Dihydromorphine	0.964	0.966	1.37E-06	0.907	0.936	9.40E-46
	Codeine	0.896	0.982	8.14E-08	0.842	0.952	7.72E-52
	Dihydrocodeine	0.969	0.981	1.12E-07	0.916	0.957	4.40E-54
Antidiabetics	Metformin	0.995	0.998	1.08E-11	0.980	0.990	1.09E-83
	Sitagliptin	0.698	0.924	4.92E-05	0.475	0.560	1.72E-09
Lifestyle chemicals and their metabolites	Caffeine	0.991	0.996	1.64E-10	0.803	0.896	5.78E-36
	1,7-dimethylxantine	0.989	0.994	4.20E-10	0.910	0.954	1.84E-52
	Nicotine	0.892	0.945	1.21E-05	0.753	0.868	2.76E-31
	Cotinine	0.978	0.989	8.58E-09	0.971	0.985	2.38E-76
NSAIDs and their metabolites	Ibuprofen	0.456	0.675	2.30E-02	0.298	0.546	5.20E-09
	2-Hydroxyibuprofen	0.964	0.981	1.10E-07	0.866	0.930	4.56E-44
	Corboxyibuprofen	0.924	0.961	2.40E-06	0.447	0.668	4.08E-14
	Naproxen	0.907	0.953	6.00E-06	0.627	0.792	1.66E-22
	O-demethylnaproxen	0.934	0.967	1.30E-06	0.616	0.785	7.46E-22
	Diclofenac	0.993	0.996	1.60E-10	0.929	0.964	2.13E-57
	2-Hydroxydiclofenac	0.875	0.936	2.32E-05	0.283	0.532	1.50E-08
Antibiotics and their metabolites	Metronidazole	0.955	0.977	2.328E-07	0.932	0.965	2.37E-58
	Hydroxymetronidazole	0.976	0.988	1.45E-08	0.949	0.974	1.20E-64
	Sylfapyridine	0.747	0.865	0.0006	0.626	0.791	1.90E-22
	N-acetyl sulfapyridine	0.813	0.902	0.00015	0.755	0.869	2.10E-31
	Sulfamethoxazole	0.915	0.956	4.135E-06	0.876	0.936	7.80E-48
	N-acetyl sulfamethoxazole	0.899	0.948	8.804E-06	0.773	0.880	4.88E-33
	Trimethoprim	0.963	0.981	9.37E-08	0.899	0.948	3.98E-50
	Ofloxacin	0.963	0.982	2.328E-07	0.883	0.939	4.90E-47
	Sulfasalazine	0.944	0.971	6.376E-07	0.275	0.891	5.11E-35
	Flucloxacillin	0.962	0.981	0.0000001	0.831	0.918	3.00E-39
	Clarithromycin	0.992	0.996	1.148E-10	0.975	0.988	6.83E-80
	N-desmethyl clarithromycin	0.643	0.705	0.015	0.185	0.431	8.70E-06
	5-Hydroxy-pyrazinoic acid	0.938	0.968	9.9E-07	0.776	0.881	3.10E-33
	Isonicotinoic acid	0.643	0.802	0.003	0.275	0.524	3.00E-08
	Ethambutol	0.972	0.986	2.8E-08	0.899	0.948	4.20E-50

		AVERAGED (ONLY SPATIAL)			FULL DATASET (SPATIAL & TEMPORAL)		
Group	Compound	R^2	r	p-value	R^2	r	p-value
Antihistamines	Fexofenadine	0.981	0.991	3.99E-09	0.947	0.973	1.58E-63
	Cimetidine	0.994	0.997	1.80E-11	0.986	0.993	9.44E-92
Cardiovascular drugs	Atenolol	0.698	0.944	4.98E-06	0.475	0.880	3.60E-33
	Irbesartan	0.940	0.968	9.90E-07	0.854	0.924	2.78E-42
	Bezafibrate	0.992	0.997	5.10E-11	0.962	0.981	6.47E-71
	Lisinopril	0.783	0.885	2.96E-04	0.717	0.847	2.27E-28
	Atorvastatin	0.968	0.986	1.70E-07	0.830	0.911	9.75E-39
WQI	Ammonia N	0.989	0.995	3.50E-10	0.600	0.774	2.20E-16
	Ortophos	0.991	0.996	1.84E-10	0.712	0.844	1.06E-21
Genetic markers	CrAssphage	0.884	0.904	1.70E-05	0.350	0.592	1.80E-08
	N1_SARS (PEG)	0.990	0.995	3.12E-10	0.266	0.516	2.57E-06
	N1_SARS (AM)	0.988	0.994	7.14E-10	0.546	0.739	2.46E-14
	NoVGI (PEG)	0.800	0.894	0.0002	0.473	0.688	1.30E-11
	NoVGII (PEG)	0.876	0.936	2.29E-05	0.154	0.392	0.00055
	NoVGII (AM)	0.902	0.950	7.69E-06	0.239	0.488	1.01E-05
	EV (PEG)	0.895	0.925	4.59E-05	0.424	0.651	3.33E-10
	EV (AM)	0.616	0.878	1.44E-08	0.061	0.904	1.56E-37
	EVD38 (PEG)	0.860	0.933	1.25E-03	0.135	0.368	1.26E-03
	EVD38 (AM)	0.616	0.785	0.0042	0.060	0.245	0.0363
Household (personal care) and industrial chemicals	Methylparaben	0.698	0.985	0.002132	0.475	0.689	3.01E-15
	Ethylparaben	0.869	0.932	2.90E-05	0.699	0.836	4.94E-27
	Propylparaben	0.609	0.780	4.60E-03	0.414	0.644	6.62E-13
	Butylparaben	0.994	0.997	3.73E-10	0.987	na	na
	Benzophenone-1	0.990	0.995	2.19E-10	0.926	0.962	1.64E-56
	Benzophenone-4	0.930	0.964	1.68E-06	0.780	0.883	1.15E-33
	Bisphenol A	0.021	0.146	6.60E-01	0.036	0.060	0.556
General physio/health markers	Creatinine	0.798	0.973	4.62E+07	0.915	0.957	1.08E-53
	8-Deoxyguanosine	0.851	0.923	5.24E-05	0.555	0.745	9.64E-19
	NHE-MA	0.976	0.988	1.44E-08	0.817	0.904	1.56E-37

R^2	r	p-value
0.95	0.9	≥0.005
1	1	<0.005
<0.7	<0.8	

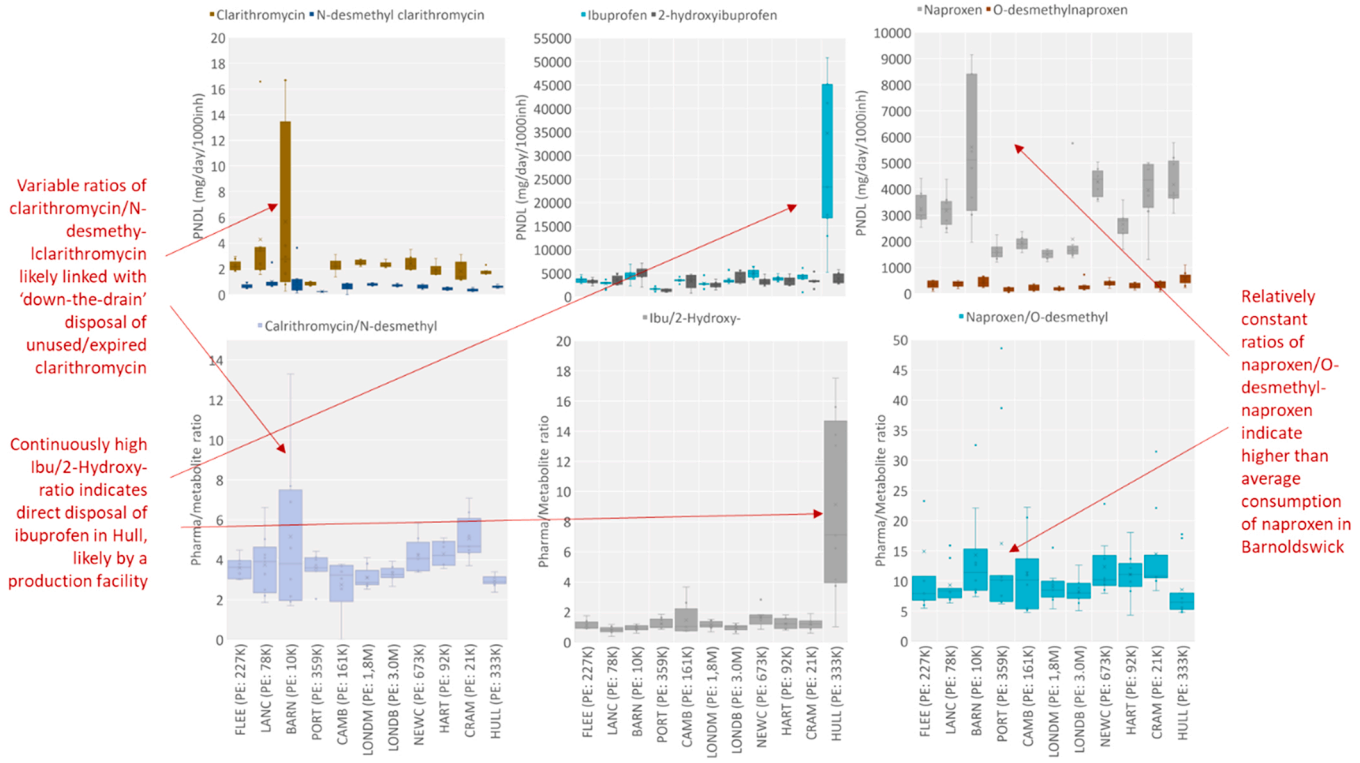

Fig. 2. Community driven pharmaceutical usage: Population Normalised Daily Loads (PNDLs) of selected pharmaceuticals their metabolites and their variable parent/metabolite ratios as indicators of pharma usage.

(Primerdesign, UK).

Adenovirus and crAssphage DNA were quantified using the Quanti-Fast SYBR and the QuantiNova Probe qPCR reagents, respectively, with 1 μg BSA in the reaction mix, as described previously [17,16].

The initial qPCR data analysis and quality control was performed using the QuantStudio Flex 6 real-time PCR software v1.7.1 (Applied Biosystems Inc., USA). The viral concentrations were expressed as gc L^{-1} RNA/DNA extract. The viral concentrations in the concentrated samples were calculated as (Eq. 3):

$$VC\ conc = \frac{\text{concentration of the nucleic acid extract} \times \text{extract volume}}{\text{volume of sample supernatant processed}} \quad (3)$$

The viral concentrations (C_{VI}, gc/mL) in the original unconcentrated samples were calculated as (Eq. 4):

$$C_{VI} = \frac{\text{concentration of the nucleic acid extract} \times \text{extract volume}}{\text{volume of sample extracted}} \quad (4)$$

Phi6 virus recoveries were calculated as (Eq. 5):

$$\frac{\text{concentration of the concentrated samples}}{\text{concentration of the unconcentrated samples}} \times 100\% \quad (5)$$

When the Phi6 control recovery was < 0.1%, the quantification was repeated with 2 μL sample/reaction, however, the reduced volumes did not affect recovery rates, suggesting little inhibition.

Daily gene loads of biological viral markers, *VIs* (gc day^{-1}), were calculated by multiplying total *VI* concentrations (gc L^{-1}) in a 24 h composite raw wastewater sample by daily wastewater flow rates (L day^{-1}). Total *VI* concentrations in raw wastewater were calculated using Eq. 6:

$$DL_{VI} = C_{VI} x V \quad (6)$$

where: C_{VI} is the total concentration of *VI* (gc L^{-1}) in influent wastewater, *V* is the volume of wastewater received by the WWTP per day (L day^{-1}).

Population normalised daily gene loads of *VIs* (gc day^{-1} $1000inh^{-1}$) were calculated using Eq. 7:

$$PNDL_{VI} = \frac{DL_{VI}}{PE_{\phi}} x 1000 \quad (7)$$

where: DL_{VI} is the daily gene load of *VI* (mg day^{-1}) in influent wastewater, PE_{ϕ} is the population estimate of ϕ which refers either to the water utility PE estimate (PE_{WW}) or a population size of patients registered in primary care (PE_{NHS}), inh indicates inhabitants.

2.4.5. Wastewater physico-chemical quality assessment (WQI markers)

Wastewater ammonium (NH_4^+) concentrations were determined colorimetrically using the salicylic acid procedure of Mulvaney (1996). Orthophosphate ($[PO_4]^{3-}$) was determined according to the molybdate blue method of Murphy and Riley (1962). All analysis was performed in a 96-well plate format using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT). Wastewater electrical conductivity (EC) was measured using a Jenway 4520 conductivity meter and pH with a Hanna 209 pH meter (Hanna Instruments Ltd., Leighton Buzzard, UK). Turbidity was measured with a HI-83414 benchtop turbidity meter (Hanna Instruments Ltd).

3. Results and discussion

Several BCIs were investigated in this multi-city WBE study, focussed on 11 WWTPs collecting wastewater from towns and cities across England (Fig. 1, Table 1) equating to a population of ~7 M people.

3.1. Wastewater flows and population equivalents

Wastewater flow rates were obtained from participating water companies. A very good fit of the linear regression model ($R^2 = 0.995$) indicated a strong linear relationship between PE and wastewater flows (Fig S2). However, it must be acknowledged that samples were collected in a short period of time (2 weeks), whilst there is an expectation that, in

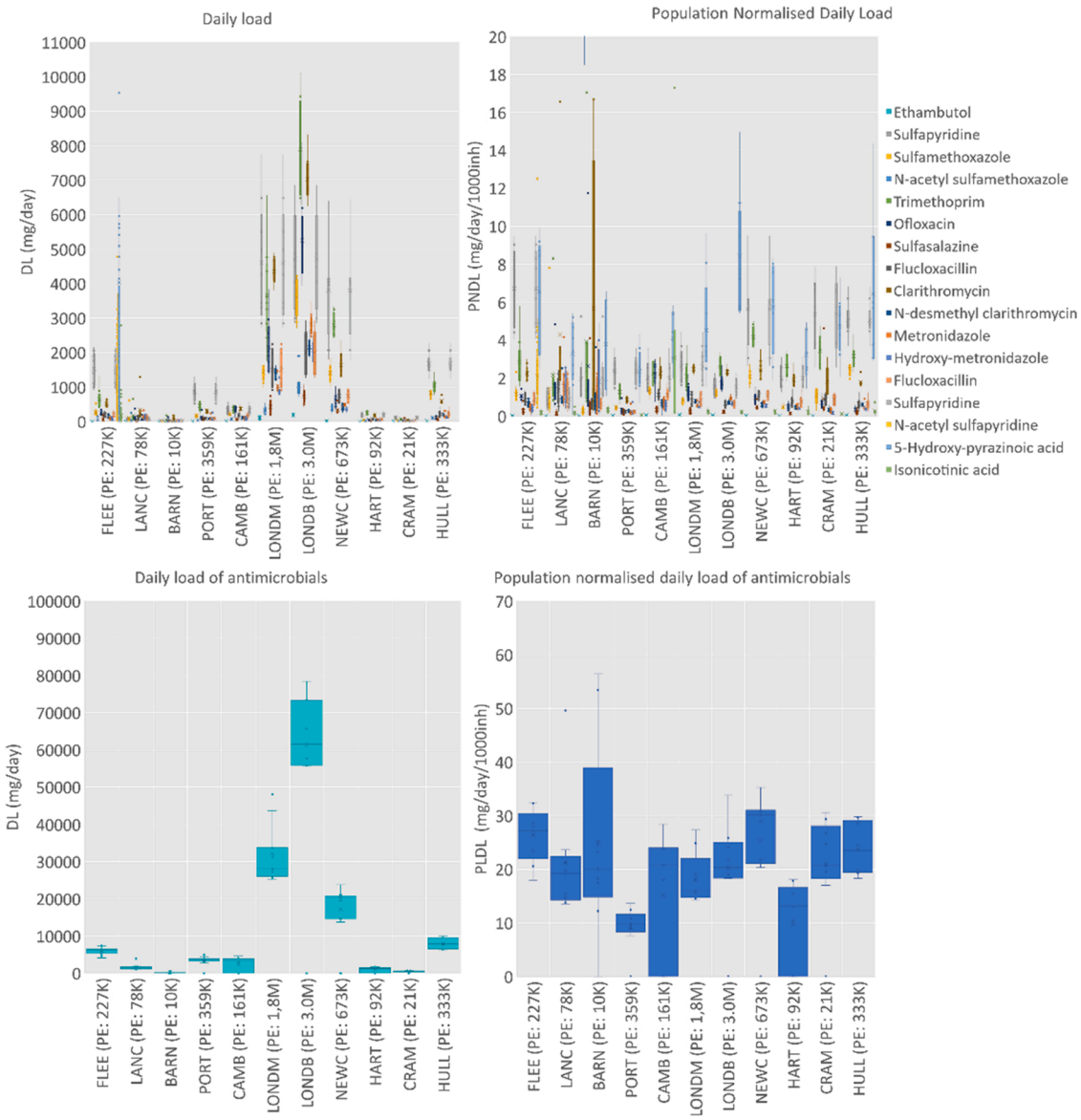

Fig. 3. Daily Loads (DLs) and Population Normalised Daily Loads (PNDLs) for antimicrobials in cities and towns in England.

longitudinal studies, higher variability in flows will be observed over longer time periods (e.g. during different seasons). From all investigated sites, only PORT did not fit this linear relationship. It is suspected that not all of the population was captured at the WWTP resulting in an underestimation of the average daily flow. PE-REG calculated from the linear regression (Fig S2) indicates that PE for flows received by PORT are 117,595 and not 359,082 as originally thought.

High diurnal variability in measured flows (measurements were taken every 15 min over a 24 h period) was observed, which reinforced the need for daily composite sampling. Low inter-day variability in measured daily wastewater flows was observed with < 10% variation on average.

Population equivalents provided by the water utilities (PE-WW) are reported in Table 1. The population estimates using GP patient registration information, PE-NHS, were also calculated, as shown in Table 1. We have used October 2021 and January 2022 datasets to coincide with the sampling period to ensure best estimates. For most of the catchments the percentage difference between these two periods is less than 1%, except CAMB WWTP, where the percentage difference is 2.2% (see SI).

We then compared the calculated PE-NHS with PE-WW provided by WWTP operators, and calculated the percentage difference between these two values, as shown in Table S2. For the PE-NHS, we have used the average value of the two time periods (October 2021 and January 2022). Among the calculated 10 locations, 8 showed negative percentage difference values, with BARN and LANC showing greater values of − 37.4% and − 24.7%, respectively, whereas CAMB showed higher positive percentage difference of 18.5%, and the remaining sites were in the range from 2.0% to − 8.3%. Further work is required to understand the factors influencing the differences (e.g. PE-NHS not accounting for visitors). Comparison of two independent PE estimates shows that PE-WW could be utilised as a reliable source of PE in the studied cities. PE-WW estimates were therefore taken forward in calculations.

3.2. BCIs in wastewater

3.2.1. Chemical markers

Several chemical markers were present in appreciable quantities, see Table 1 and Figs. 2–5. Chemical markers, including pharmaceuticals, lifestyle chemicals, industrial and personal care products as well as endogenously formed health markers, were present at all WWTPs with variable daily loads (Figs. S3–7). LONDB&M followed by NEWC receive the highest daily loads, which are, to a large extent, driven by the size of

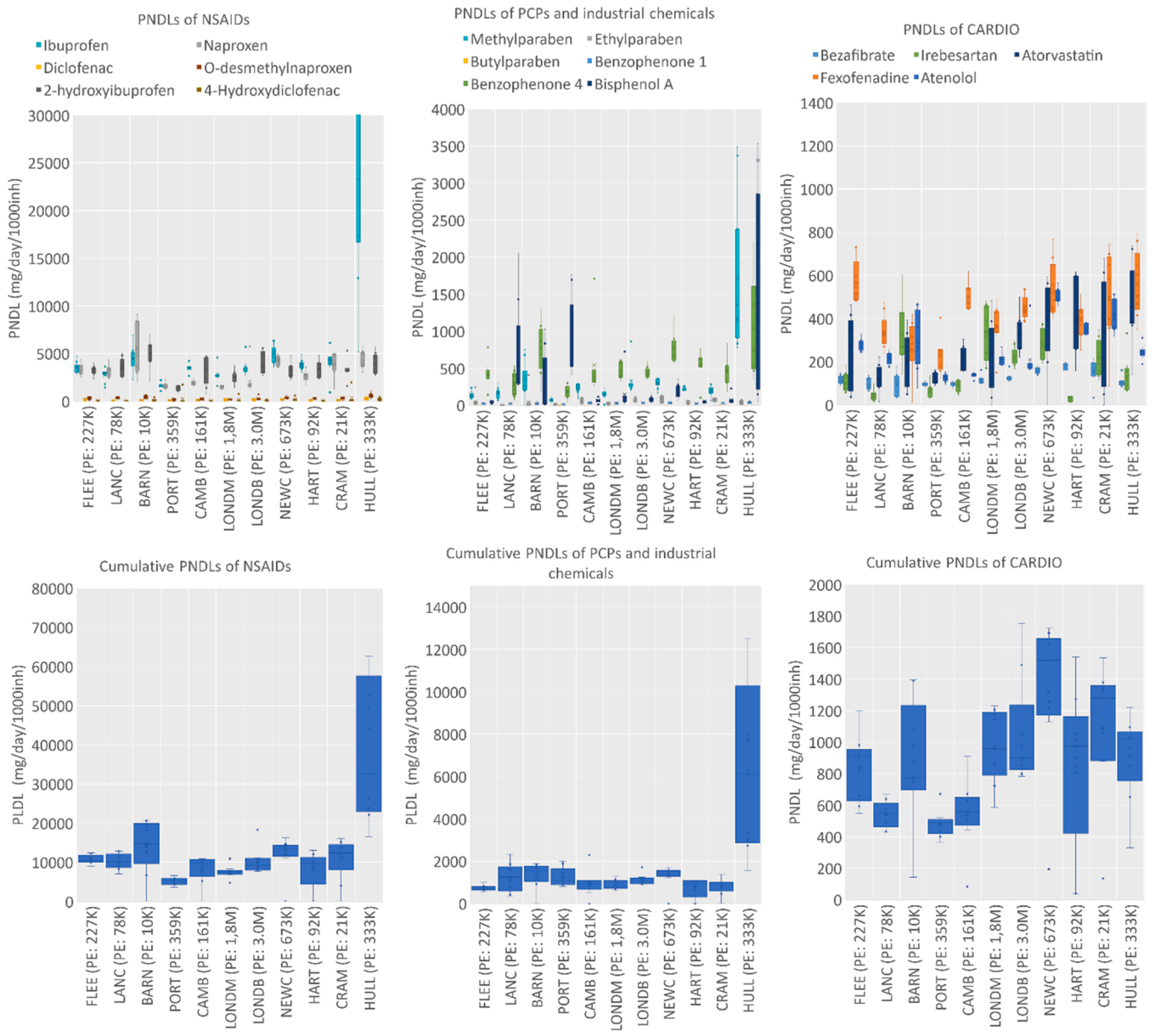

Fig. 4. Population Normalised Daily Loads (PNDLs) for NSAIDs, CARDIO, their metabolites, and industrial/PCP chemicals (DLs are available in SI) in cities and towns in England.

population contributing to wastewater (especially NCDs). This is clearly seen when double normalisation (to wastewater flows and population size) is applied (see Daily Load, DLs and Population Normalised Daily Loads, PNDLs, in Figs. S3–7). However, there are several exceptions providing insights into chemical intake that can inform either disease status in various communities or unintentional exposure to hazardous chemicals: e.g. very high PNDLs of ibuprofen in HULL and Bisphenol A (BPA) in HULL, LANC and PORT. While high PNDL of ibuprofen is likely linked with direct disposal of this pharmaceutical, high BPA levels are generally related to industrial discharge. Indeed, high levels of ibuprofen in HULL (Fig. 2) were confirmed as resulting from direct disposal of ibuprofen, as seen when examining ibuprofen/2-hydroxyibuprofen ratios (ibu-/2-hydroxy-). While the average ibu-/2-hydroxy- ratio across 9 WWTPs is 1.2 ± 0.5, which is in line with our previous work (Kannan et al., 2022), ibu-/2-hydroxy- levels in HULL were 9.1 ± 5.6, which indicates levels higher than those expected from metabolism. As the higher ibu-/2-hydroxy- ratio persisted throughout the sampling period, it is assumed that ibuprofen leaches from e.g. production facilities, rather than being linked with direct household disposal into the sewer system [32,33,31]. If the latter was the case, only a short interval (one day) ibu-/2-hydroxy- peak would have been observed. The above observations indicate a clear potential of WBE to understand spatiotemporal patterns of pharmaceutical usage by communities.

There is, in general, an expected greater variability in antibiotics usage across communities (Fig. 3). This is due to the intermittent nature of antibiotics' usage in contrast to NCD pharmaceuticals that treat chronic conditions. Higher PNDLs were observed on several occasions, e.g. several times higher than average clarithromycin PNDLs. An inspection of clarithromycin/N-desmethylclarithromycin ratios (Fig. 2) indicates a case of direct disposal rather than higher usage. On the other hand, relatively constant naproxen/O-desmethylnaproxen ratios associated with higher-than-average naproxen PNDLs indicate higher usage rather than direct disposal. These observations indicate a critical need for the inclusion of endogenously formed metabolites of chemical targets in WBE studies to fully understand exposure patterns.

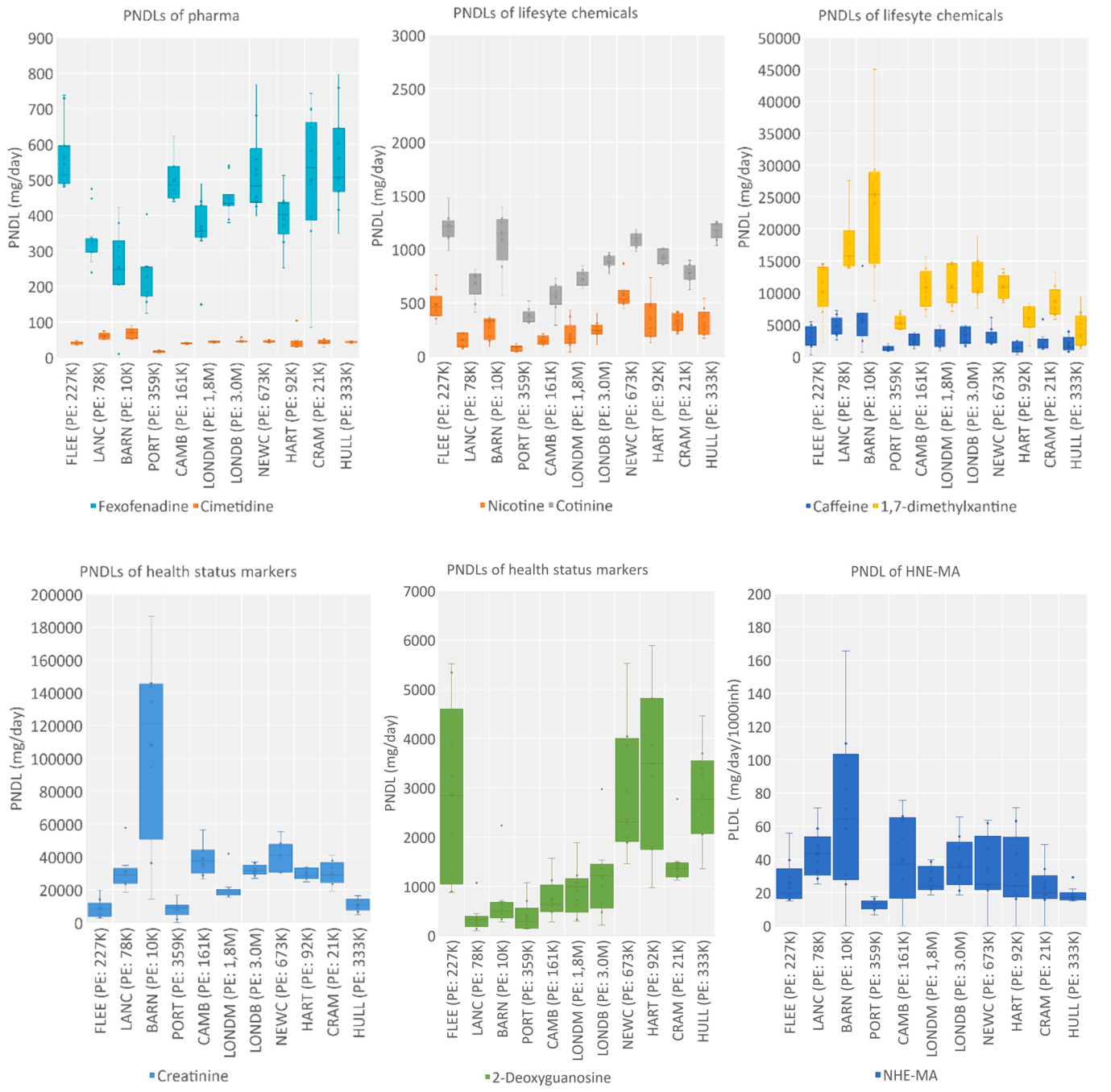

Fig. 5. Population Normalised Daily Loads (PNDLs) for lifestyle chemicals, high usage pharmaceuticals, and their metabolites, as well as general endogenous markers (DLs available in SI) in towns and cities in England.

An importance for tracking endogenous health markers such as 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA, an oxidative stress marker) as a generic marker of health status in communities is clearly observed in Fig. 5. Increased levels of HNE-MA were seen at BARN. They coincided with higher-than-average paracetamol usage (Fig. 5) and SARS-CoV-2 prevalence (Fig. 6).

3.2.2. *Viral markers targeted in wastewater*

The two methods used for sample concentration for virus detection (BE-PEG and Amicon) resulted in similar detection rates and relative concentration patterns, however, some differences were seen for individual viruses. Overall, statistical analysis suggested that both methods are suitable for virus detection in wastewater with the Amicon method performing slightly better when the samples contain high concentrations of ammonium [19]. Neither method was able to detect Influenza B virus, HIV, Hepatitis A, B and C viruses in any of the wastewater samples. Measles, influenza A viruses and rotavirus were only detected sporadically at 1–6 sites. Due to the lack of quantitative standards, the RSV-A/B results were only qualitative, however, RSV-A/B was positively identified in $>80\%$ of samples. CrAssphage, enteroviruses, including the D68 variant, as well as norovirus GI and GII were detected at all sites, which shows their endemic nature, but at a variable frequency. This is similar to the observations for SARS-CoV-2; samples were collected in November 2021 during a SARS-Cov-2 high prevalence period attributable to the Delta variant, with the highest gc d^{-1} $1000inh^{-1}$ observed in LOND and NEWC.

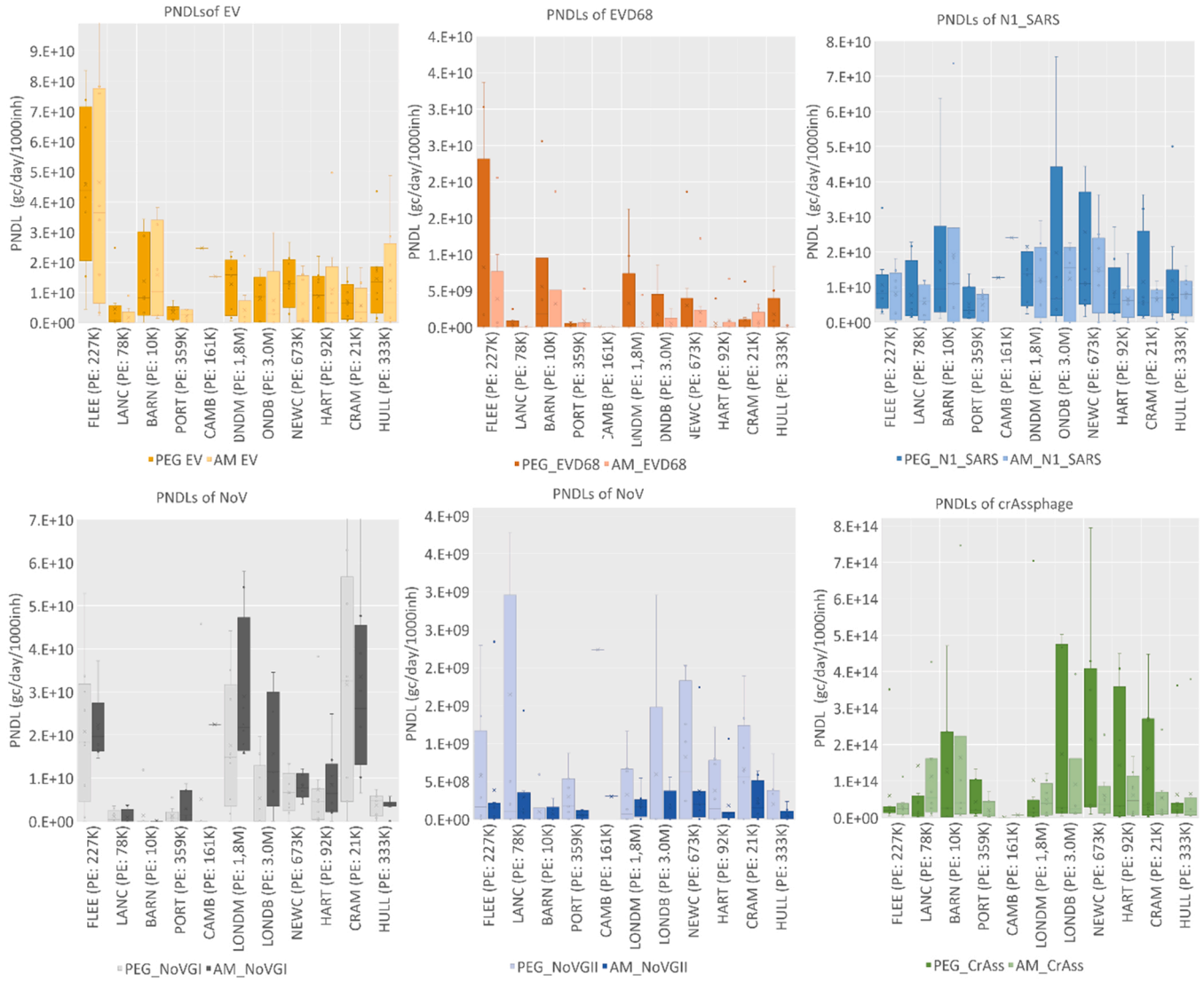

Fig. 6. Daily Loads (DLs) and Population Normalised Daily Loads (PNDLs) of viral markers and the faecal marker virus crAssphage in towns and cities in England.

It is interesting to note that the overall cumulative viral load, similarly to chemical load, was recorded to be the highest at LOND WWTPs (the largest and third largest treatment works in the UK), and NEWC WWTP, corresponding to sites with the largest population contributions to the wastewater load (Figs. S8). However, normalisation to population size revealed patterns that are likely linked with differential prevalence of viruses across communities. For example, enteroviruses (including D68) gc d^{-1} $1000inh^{-1}$ were relatively constant across all sites except FLEE, which received much higher daily loads, likely due to higher viral prevalence at the time of sampling. Interestingly, norovirus was also very prevalent in this community in comparison with SARS-CoV-2, which was relatively constant across all 11 urban areas due to high prevalence of Delta variant during sampling, as discussed above. In contrast, norovirus GI and GII showed different prevalence with GI being the most prevalent in LONDC (North East England), and GII in LANC (North West England) (Fig. 6).

3.2.3. Water quality indicators

WQIs were tested to have a broader understanding of general wastewater characteristics, such as pH, electrical conductivity and turbidity, as they can provide information of key wastewater characteristics that might be affecting extraction of BCIs from wastewater. As seen in Figs. S9, no anomalies were observed in analysed wastewater samples with pH, turbidity, conductivity, ammonium and orthophosphate being on average (± SD): 7.5 ± 0.2, 95.4 ± 58.5 NTU, 1356 ± 982 μS cm^{-1}, 9.9 ± 9.0 g/day/inh, 0.9 ± 0.5 g/day/inh, respectively.

3.3. Comparison of inter-city BCI daily loads as a function of urban population size

Linear regression was performed to describe the statistical relationship between daily BCI loads and population size served by selected WWTPs, with R^2 in most cases being > 0.9 showing very good fit of the model. Pearson's r being on average > 0.9, indicated a strong positive linear correlation between averaged BCI loads across all sampling days and individual BCI daily loads and PE. The *p*-value obtained for all but a few BCIs was < 0.001 proving further evidence of a significant correlation between BCIs loads and PEs described by the model (Table 1).

The results clearly indicate that there is a strong positive correlation between several BCIs and PE with relatively few BCI groups showing much weaker correlations. To account for this, BCIs were divided in three main groups (Table 1):

Group 1. : BCIs with the strongest correlations (R^2 >0.9, r > 0.9) and low inter-day variability with usage independent of city functions. These are mostly, high usage NCD pharmaceuticals with multi-spectrum applications focussed on chronic disease and high prescription patterns:

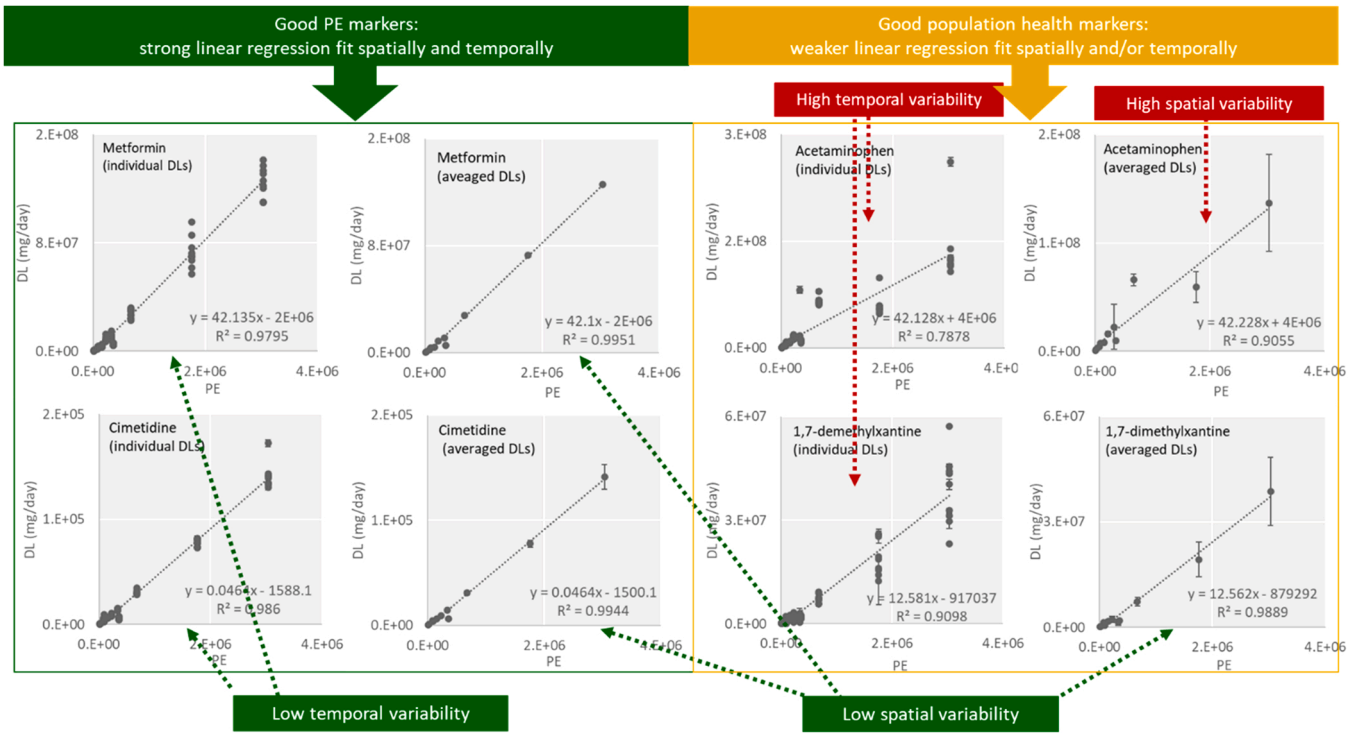

Fig. 7. DLs of BCIs and their spatial and temporal variability.

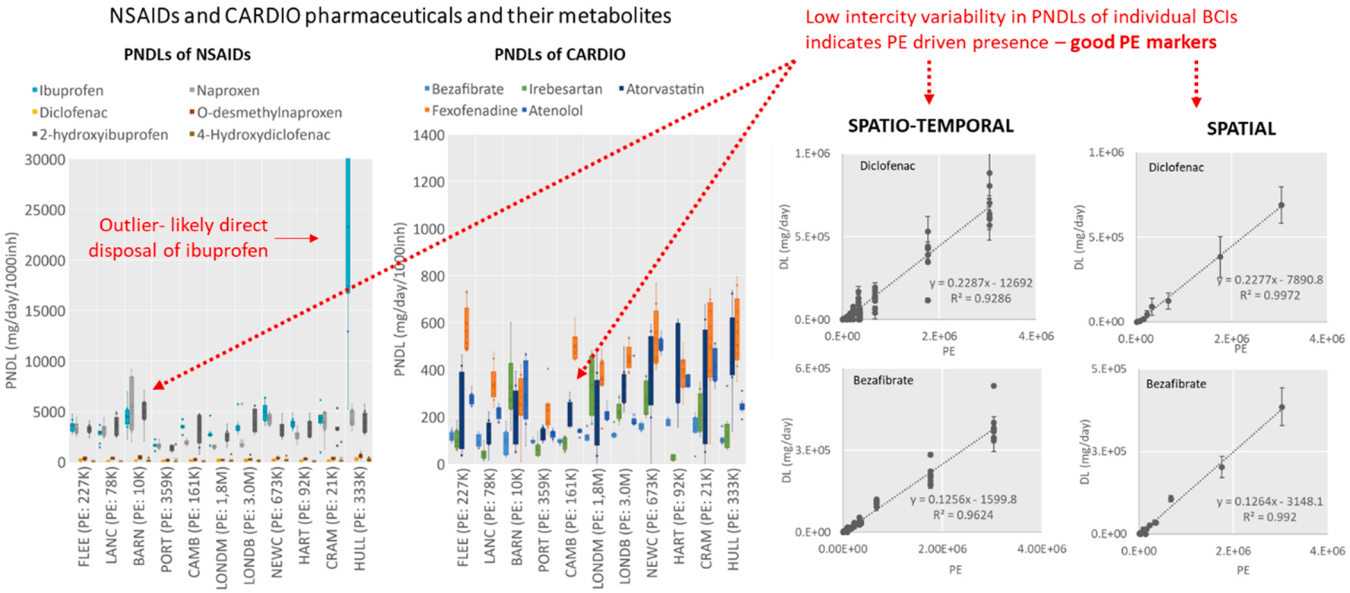

Fig. 8. PNDLs and DLs of NSAIDs and CARDIO pharmaceuticals - potential markers of population size.

metformin, cimetidine, diclofenac, bezafibrate and most importantly lifestyle chemicals (e.g., nicotine, caffeine and their metabolites).

Group 2: BCIs with medium-high correlation (0.9 <R2 >0.7 and 0.9 <r > 0.8), higher inter-day variability in daily loads, and with usage of seasonal nature. These are mostly cardiovascular pharmaceuticals, NSAIDs, antibiotics and WQIs.

Group 3: BCIs with lower correlations (R2 <0.7, r < 0.8) with usage dependent on city function and/or subject to random incidents, e.g. chronic or acute chemical exposure due to low occupational standards, an outbreak of an infectious disease. These are viral markers as well as industrial chemicals such as BPA.

3.3.1. *Group 1: BCIs with constant per capita usage*

Most BCIs showed a strong positive linear correlation between total averaged BCI loads (averaged for individual WWTPs across all nine sampling days) and PE indicating their low spatial variability. However, only a very few BCIs showed the same strong positive linear correlation between non-averaged DLs and PE (an indication of their low temporal variability) (Figs. 7–9). These are: antidiabetic metformin, NSAID diclofenac, histamine H_2 receptor antagonist cimetidine, cardiovascular bezafibrate, and metabolites of lifestyle chemicals: 1,7-dimethylxantine and cotinine. These BCIs are recommended as potential PE indicators. It is expected that Group I BCIs will show higher spatial and temporal variability in the context of global usage. This is due to different disease

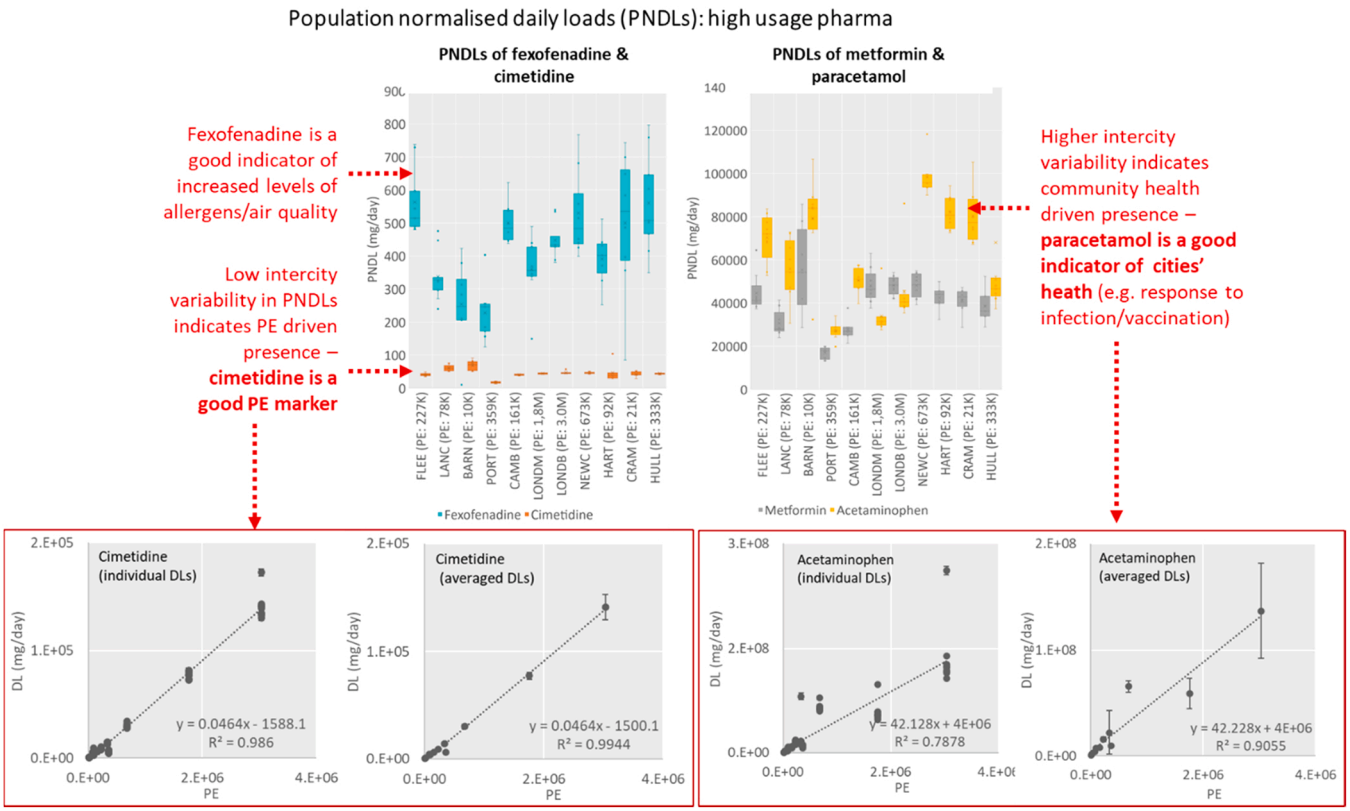

Fig. 9. PNDLs and DLs of lifestyle chemicals and high usage pharmaceuticals - potential indicators of community's health.

diagnostics, prevalence, and prescription patterns, as well as drug availability. Possible temporal variabilities should also be taken into account in future longitudinal (time series) studies. Their utility as PE markers has, therefore, to be evaluated in-situ at study locations. Some Group I BCIs could be also considered for disease prevalence as well as pharmaceutical intervention strategies, globally.

3.3.2. Group 2: BCIs with variable per capita usage

3.3.2.1. Pharmaceuticals as proxy for wider disease prevalence. PNDLs of group 2 pharma BCIs can provide invaluable information on community-wide pharmaceutical consumption, which can then be used as a tool to provide information on pharmaceutical prescription/usage compliance, and most importantly, it can serve as a proxy for the prevalence of certain diseases. Figs. S3–5 show DLs and PNDLs for individual BCIs and total DLs/PNDLs for each group. The weak fit of the linear regression model indicates that DLs of pharma are not necessarily only driven by PE in different catchments, both spatially and temporarily. This indicates that other factors come into play including disease prevalence as well as socioeconomic status of surveyed communities. Indeed, while prescription data can provide information on prescription patterns, only WBE can inform actual use at a community level and evaluate pharma usage compliance. This is of particular importance in the case of pharmaceuticals that can be sourced over the counter (e.g. NSAIDs and antihistamines), such as those used for pain treatment or those that are not reported in official statistics (e.g. tuberculosis drugs such as ethambutol as seen in Fig. 3).

Paracetamol is a good example of an over-the-counter medication with variable temporal usage triggered by public health status, e.g. higher covid prevalence or vaccination during November 2021 sampling period (Fig. 9), as discussed in Section 3.2.1. Fexofenadine, an antihistamine drug, is another interesting example. It shows high variability both spatially and temporarily (Fig. 9). Further work needs to be undertaken via data triangulation, for example, with public health and environmental data on air quality in surveyed communities to see associations between fexofenadine levels and allergy drivers.

3.3.2.2. Viral markers. PNDLs of virus markers were found to be highly variable (Fig. 10). Depending on their nature: i.e. endemic or emerging threat, they fall in either Group 2 or 3. SARS-CoV-2 is a good example. Being very prevalent in communities nationwide during sampling, its presence in wastewater was to a large extent community size driven. The same applies to crAssphage, which is very prevalent in urban communities [27]. In contrast, norovirus and enterovirus showed much higher variability in prevalence across all sites investigated, with clear cases of localized outbreaks in some cities while maintaining low prevalence in other locations. This may be due to high restriction levels in mixing of people, regular disinfection and general better hand hygiene due to COVID pandemics, which greatly reduce the spread of enteric pathogens as well [4].

3.3.3. Group 3: BCIs as proxy for city function

Group 3 biomarkers are those with lowest correlations between PNDLs and PE. This is because their presence in wastewater is driven by city function and/or random in nature biological or chemical disposal events, and not general community's chemical consumption. As can be seen in Fig. 10, significantly higher PNDLs of BPA (a regulated endocrine disruptor) were recorded in HULL, PORTS, BARN and LANC indicating higher industrial presence of BPA (plastics) dependent industries. BPA-sulphate would have to be quantified via retrospective analysis to see if higher BPA levels are also linked with higher occupational exposure (see [32,33,37,36,31] for further details). There were also some significant variations of benzophenone-4 PNDLs observed (Fig. 11), which warrants further investigation regarding industries using this chemical in the studied sewerage catchments. As discussed above, norovirus and enterovirus also belong to this group due to high variability of localized nature.

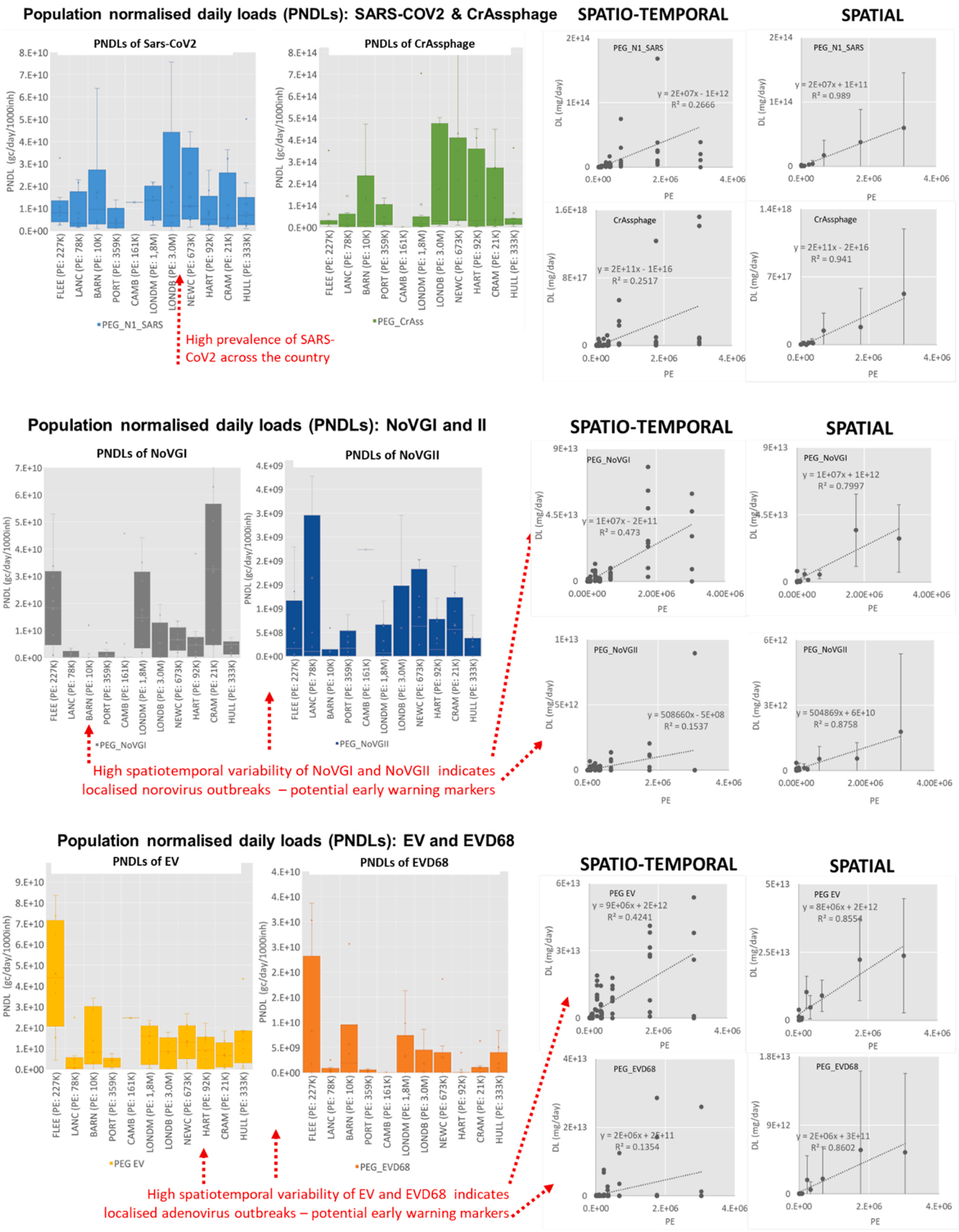

Fig. 10. PNDLs of viral targets.

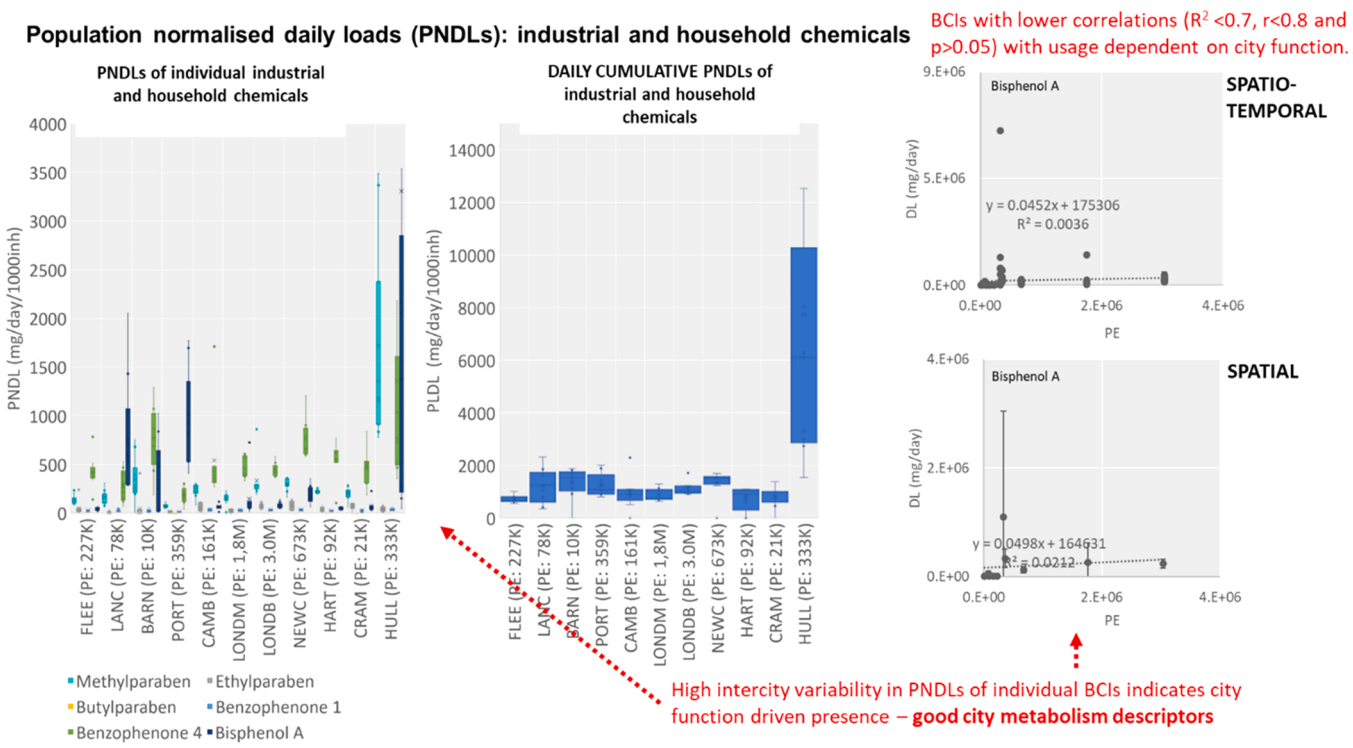

Fig. 11. PNDLs of household and industrial chemicals.

4. Conclusions

Our study tested the hypothesis that a biochemical burden in any given catchment (derived from wastewater from this catchment and measured with WBE tools) is driven by population contributing to this catchment, socioeconomics and city's function, which could enable management strategies aimed at increased environmental and public health in this catchment. Such an approach is particularly promising in the context of One Health as it enables a holistic understanding of city's metabolism encompassing all the activities of a city in a single model: from lifestyle choices, through to health status and exposure to harmful chemicals and pathogenic organisms as well as effectiveness of implemented management strategies.

Several groups of BCIs were the subject of investigation in an inter-city system including 10 cities/towns from across England. Biochemical mining of wastewater for BCIs was undertaken to understand spatio-temporal speciation of BCIs.

The key conclusions are as follows:

1. PNDLs of many chemical markers were found to be driven by the size of population contributing to wastewater (especially NCDs). However, there are several exceptions providing insights into chemical intake that can inform either disease status in various communities or unintentional exposure to hazardous chemicals: e.g. very high PNDLs of ibuprofen in HULL resulting from its direct disposal (confirmed by ibuprofen/2-hydroxyibuprofen ratios) and BPA in HULL, LANC and PORT related to likely industrial discharge. Relatively constant naproxen/O-desmethylnaproxen ratios associated with higher-than-average naproxen PNDLs indicate higher usage rather than direct disposal. These observations indicate a critical need for the inclusion of endogenously formed metabolites of chemical targets in WBE studies to fully understand exposure patterns.
2. An importance for tracking endogenous health markers such as 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA, an oxidative stress marker) as a generic marker of health status in communities is clearly observed. Increased levels of HNE-MA were seen at BARN. They coincided with higher-than-average paracetamol usage and SARS-CoV-2 prevalence.
3. PNDLs of virus markers were found to be highly variable. SARS-CoV-2, being very prevalent in communities nationwide during sampling, its presence in wastewater was to large extent community driven. The same applies to crAssphage, which is very prevalent in urban communities. While norovirus and enterovirus showed much higher variability in prevalence across all sites investigated, with clear cases of localized outbreaks in some cities while maintaining low prevalence in other locations. This may be due to high restriction levels in mixing of people, regular disinfection and general better hand hygiene due to COVID pandemics, which greatly reduce the spread of enteric pathogens as well.

Comprehensive, multi-target analysis of biomarker groups in an inter-city system is critical to allow for identification of key public health drivers, resulting general community health status (often linked with particular socioeconomic circumstance driving specific disease prevalence) and has a strong potential to enable city-system focussed public health interventions.

Environmental implications

This manuscript showcases results from a large scale and comprehensive wastewater-based epidemiology (WBE) study focussed on multi-biomarker suite analysis of both chemical and biological determinants in 10 cities and towns across England equating to a population of ~7 million people. Multi-biomarker suite analysis, describing city metabolism, can provide a holistic understanding to encompass all of human, and human-derived, activities of a city in a single model: from lifestyle choices (e.g. caffeine intake, nicotine) through to health status (e.g. prevalence of pathogenic organisms, usage of pharmaceuticals as proxy for non-communicable disease, NCD, conditions or infectious disease status), and exposure to harmful chemicals due to environmental and industrial sources (e.g. pesticide intake via contaminated food and

industrial exposure).

CRediT authorship contribution statement

Barbara Kasprzyk-Hordern: Conceptualization, Methodology (experimental design, data processing), Formal analysis, Data curation, Writing – original draft, Writing – review editing, Supervision, Project administration, Funding acquisition, Resources. **Natalie Sims:** Methodology (sample preparation, LCMS analysis), Writing – review editing. **Kata Farkas:** Methodology (sample preparation, viral analysis), Writing – review editing. **Kishore Jagadeesan:** Methodology – Methodology (PE NHS calculation), Writing – review editing. **Kathryn Proctor:** Methodology (sample preparation), Writing – review editing. **Matthew Wade:** Conceptualization, Writing – review editing. **Davey Jones:** Conceptualization, Writing – review editing, Supervision, Project administration, Funding acquisition, Resources.

Declaration of Competing Interest

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

Data Availability

Data is available in Si.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.130989.

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