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Technical Report

Mass and toxic loads of environmentally relevant chemicals in two wastewater treatment plants in Greece

Wide-scope target analysis, suspect screening and ecotoxicological risk assessment

Nikolaos S. Thomaidis, Georgios Gkotsis, Reza Aalizadeh, Rallis Lougkovois, Triantafyllos-Dimitrios Gerokonstantis, Christoforos Bouzoukas, Antigoni Konomi, Katerina Ragazou, Marios Kostakis, Georgios Gourgouletis

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List of Abbreviations

AES	Alcohol Ethoxy Sulfates
AI	Artificial Intelligence
ASW	Artificial Sweeteners
bbCID	broadband Collision Induced Dissociation
CCS	Collision Cross Section
CTC	Coffee and Tobacco related Chemicals
DL	Deep Learning
DRG	Drugs of Abuse
EEL	Wastewater Treatment Plant - Larissa
EPR	Extended Producer Responsibility
ESI	Electrospray Ionization
EYDAP SA	Athens Water Supply and Sewerage Company
HMT	Heavy Metals
HRMS	High Resolution Mass Spectrometry
ICP	Inductively Coupled Plasma
IND	Industrial Chemicals
IS	Internal Standard
KELPS	Wastewater Treatment Plant - Psyttallia
LAC	Laboratory of Analytical Chemistry
LAS	Linear Alkylbenzene Sulfonates
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
M&TPs	Metabolites and Transformation Products
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
PASEF	Parallel Accumulation Serial Fragmentation
PCP	Personal Care Products
PFAS	Per- and Polyfluoroalkyl Substances
PHR	Pharmaceuticals
PPP	Plant Protection Products
QSAR	Quantitative structure–activity relationship
QTOF	Quadrupole-Time of Flight
QTRAP	triple Quadrupole-linear Ion Trap
RTI	Retention Time Index
SPE	Solid Phase Extraction
SRF	Surfactants
TIMS	Trapped Ion Mobility Spectrometry
TAC	Tire Additive Chemicals

VET	Veterinary Drugs
WWTP	Wastewater Treatment Plant

1. Introduction

1.1 Background and problem definition

Urban wastewater discharges constitute a major pathway for the release of diverse classes of chemicals into aquatic environments across Europe. These compounds include pharmaceuticals, industrial chemicals, plant protection products, surfactants, food additives, per- and polyfluoroalkyl substances (PFAS), and other anthropogenic chemicals. Recently, EU Directive 2024/3019 was established for European Member States, replacing Urban Wastewater Treatment Directive 91/271/EEC. The recently adopted Directive intensifies the requirements for monitoring, treatment, and extended producer responsibility for pollutants in urban wastewater plants.

Specifically, EU Directive 2024/3019 establishes a modernized and more comprehensive framework for urban wastewater treatment across Member States, introducing mandatory monitoring of pollutants in both influent and effluent wastewater, as well as receiving waters. Moreover, it requires the implementation of advanced tertiary treatment capable of removing a broad range of pollutants, reflecting growing concerns about the complex chemical mixture, which ends up in aquatic environments. Another key point of the Directive is the Extended Producer Responsibility (EPR) policy, under which producers - primarily in the pharmaceutical and cosmetic sectors - are obligated to cover at least 80% of the associated treatment and monitoring costs, designating them as principal contributors to the remediation of chemical loads in urban wastewater. There is also a potential extension to other industries depending on national transposition.

Several monitoring studies conducted in Greece on urban wastewater, surface waters, drinking-water reservoirs, and rivers impacted by industrial and agricultural activities, reveal that they are highly exposed to a wide range of chemicals, such as industrial chemicals, plant protection products, artificial sweeteners, surfactants, lifestyle-related chemicals, and pharmaceuticals. The highest concentrations (up to hundreds of μg/L) are typically observed for artificial sweeteners, surfactants, and industrial chemicals. Pharmaceuticals are generally determined at lower concentration levels (ng/L to a few μg/L).

Extensive monitoring campaigns have been conducted at the Psyttalia Wastewater Treatment Plant (WWTP), which is one of the largest WWTPs globally and the primary facility serving the Attica region. These studies demonstrate that pollutants originate from highly diverse sources, such as chemical industries, food industries, agricultural activities, pharmaceutical industries, hospital effluents, as well as human discharges. Over the past 15 years, the Laboratory of Analytical Chemistry (LAC), in collaboration with the Athens Water Supply and Sewerage Company, have performed annual sampling campaigns in Psyttalia WWTP for extensive chemical monitoring. In these studies, LAC employs state-of-the-art analytical methodologies and chemometric tools, using mass spectrometric techniques - LC-MS/MS and LC/GC-HRMS - and comprehensive wide-scope target and suspect screening approaches. These monitoring surveys have shown that, even

in WWTP with substantial pharmaceutical loading, the highest concentrations generally correspond to industrial pollutants (e.g., 1H-benzotriazole), surfactants (LAS, AES), artificial sweeteners, and food preservatives.

Early investigations of urban wastewater in Athens [1] used advanced HRMS-based integrated targeted and non-targeted screening approaches, revealing the identification of 34 previously undetected compounds, including pharmaceutical metabolites, nicotine derivatives, and antibiotics. These studies also enabled the first identification of entire homologous series of surfactants - particularly linear alkylbenzene sulfonates (LAS) and alcohol ethoxy sulfates (AES) - at notably high concentrations, highlighting the substantial and continuous contribution of detergents and cleaning products to the overall pollutant load. The insights gained from these pioneering HRMS applications laid the foundation for subsequent large-scale monitoring programs in Greece (e.g. Asopos river basin, drinking-water reservoirs, coastal water) and Europe, which significantly broadened analytical coverage to more than 3,000 targeted analytes and over 10,000 suspect compounds.

A milestone in the wastewater monitoring was the development of an HRMS-based target database, including more than 2,000 compounds, including pesticides, surfactants, industrial chemicals, pharmaceuticals, and food preservatives [2]. This database enabled the wide-scope target screening of wastewater samples, revealing that pharmaceuticals such as carbamazepine, metoprolol, diclofenac, and hydrochlorothiazide were detected at tens to hundreds of ng/L, while industrial compounds like 1H-benzotriazole reached a higher order of magnitude concentrations (e.g. 3.36 to 13.5 μg/L).

Time-trend analyses at the Psyttalia WWTP demonstrated that artificial sweeteners, such as sucralose, acesulfame, cyclamate, and saccharin occur at high and temporally stable concentrations [3]. Recent monitoring data confirm these findings across WWTPs and drinking-water reservoirs, with mean concentrations typically in the low-to-mid μg/L range and maximum values exceeding 90.0 to 100 μg/L. These substances are highly resistant to conventional biological treatment, serve as robust indicators of human activity, and contribute substantially to the overall pollutant burden, despite being unrelated to the pharmaceutical industry.

Therefore, both pharmaceutical and non-pharmaceutical compounds, as well as their metabolites and transformation products, are detected in wastewater and can present similar levels of environmental relevance and risk. While pharmaceuticals are important from environmental perspective, other substances and chemical classes like surfactants, several unclassified industrial chemicals, plasticizers and plastic-related compounds, food preservatives, pesticides, artificial sweeteners, and drugs of abuse contribute substantially to overall pollutants' loads and ecotoxicological pressures, as reflected in recent nationwide datasets.

Moreover, industrial chemicals, including phthalates, PFAS, phenols, benzotriazoles, benzothiazoles and flame retardants, originate from diverse manufacturing and consumer product uses. Pharmaceuticals and personal care products, while present, represent only one pollutant category and are often of lower priority relative to industrial or agrochemical contaminants. Monitoring of EYDAP drinking-water reservoirs showed that priority substances under Directive 2013/39/EU extend beyond pharmaceuticals. These substances are clearly linked to industrial, and consumer uses rather than pharmaceuticals.

Finally, a recent study by Lougkovois et al. (2025), applying LC-TIMS-HRMS to assess the chemical impact of the "Daniel" extreme flood event in Thessaly, screened over 2,300 organic compounds in seawater, and sediment samples [4]. These results highlight the amplification of chemical pressures due to agricultural runoff, urban discharges, flood-related releases, and industrial inputs.

1.2 Aim of the technical report

This study aims to comprehensively characterize the chemical fingerprint of influent and effluent wastewater from the **Psyttalia** and **Larissa** WWTPs using highly sensitive analytical methodologies, state-of-the-art instrumentation, and advanced chemoinformatic tools. The applied methodologies enable the detection of thousands of substances, encompassing a broad array of priority substances identified through policy frameworks and research studies. Analysis of influent wastewater provides valuable insights into societal consumption and disposal patterns, while evaluation of the Psyttalia WWTP - one of the largest facilities in Europe and globally - allows assessment of its removal efficiency for both detected and tentatively identified pollutants.

This reports summarizes the data included in a recent publication of our group [5] after the request of The Panhellenic Association of Pharmaceutical Industries (Πανελλήνια Ένωση Φαρμακοβιομηχανίας -Π.Ε.Φ.-), Hellenic Association of Pharmaceutical Companies (Σύνδεσμος Φαρμακευτικών Επιχειρήσεων Ελλάδος -Σ.Φ.Ε.Ε.-) and Association of Self-Care Medicines Companies (Σύνδεσμος Εταιρειών Φαρμάκων Ευρείας Χρήσης -Ε.Φ.Ε.Χ.-). The associations requested additional experimental data of this study, which has already been published not only in the main body of the article but mostly in the supplementary information files associated in the publication and can be retrieved in the following link: https://doi.org/10.1021/acs.estlett.6c00733 [5].

2. Objectives

The specific objectives of the study were as follows:

a) determination of **250 pharmaceuticals, drugs of abuse, their metabolites and transformation products (M&TPs)** in wastewater samples using a highly sensitive LC-MS/MS analytical methodology.
b) determination of **44 PFAS** in wastewater samples using a highly sensitive LC-MS/MS analytical methodology.
c) determination of **20 tire additive chemicals** in wastewater samples using a highly sensitive LC-MS/MS analytical methodology.
d) elemental analysis of 20 metals in wastewater using ICP-MS.
e) the determination of **more than 2,500 environmentally relevant chemicals** in wastewater samples using a comprehensive LC-IMS-HRMS-based wide scope *target screening* workflow.
f) identification and semi-quantification of **plastic-related chemicals, cosmetics, and surfactants** in wastewater samples using a comprehensive LC-IMS-HRMS-based *suspect screening* workflow (**>3100 chemicals**).
g) **ecotoxicological risk assessment** using experimental PNEC values and calculation of **%∑Toxic Loads per chemical class**.

3. Samples

The samples, analyzed in the context of the present study, were gathered from two different demo sites: Psyttalia Wastewater Treatment Plant (KELPS), Athens, Greece (**Figure 1**) and Larissa Wastewater Treatment Plant (EEL), Larissa, Greece. KELPS is the main WWTP in the wider Attica region, with an average incoming flow of approximately 730,000 m³ per day. It is one of the largest WWTPs at European and international level, with a capacity equivalent to a population of 5,600,000 inhabitants. EEL is one of the main WWTPs in Thessaly, Greece, which is an agricultural area that was highly impacted following extreme flood events in 2023. The EEL has a maximum incoming flow of average 42,000 m³ per day and a maximum capacity equivalent to a population of 227,500 inhabitants.

KELPS facilities include preliminary wastewater treatment at Akrokeramos (Attika mainland), involving debris removal, grill screening, grit removal and odor control unit. Pretreated wastewater is transported via underwater pipelines to Psyttalia island. The wastewater treatment continues in the primary sedimentation tanks, where primary sludge is collected. Subsequently, the primary-treated wastewater undergoes advanced secondary biological treatment using the activated sludge process to remove the organic load and significantly reduce nitrogen levels. This treatment takes place at the biological level, which consists of bioreactors and final sedimentation tanks, where biological sludge sedimentation clarifies the treated wastewater. The final effluent from the KELPS is discharged into the Saronic Gulf through an underwater deep-diffusion pipeline system [6, 7].

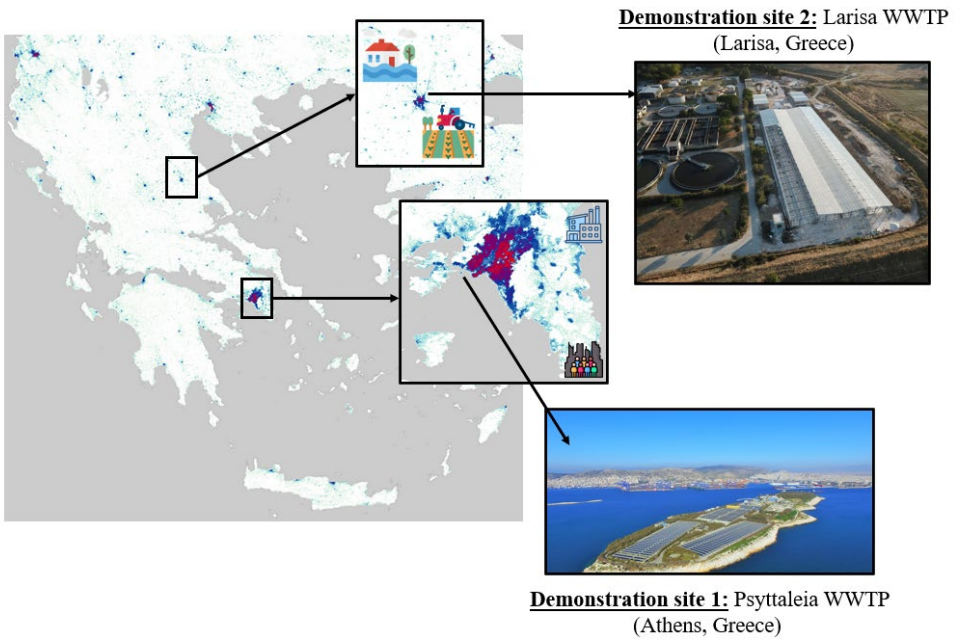

Figure 1: Demonstration sampling sites of this study and related activities in these regions.

EEL facilities include preliminary and primary treatment units for grill screening, grit removal, and sedimentation, respectively, followed by biological treatment processes for the removal of organic load and nutrients. Supporting facilities comprise aeration tanks, secondary clarifiers, sludge thickening and dewatering systems, pumping stations, and odor control units. The final effluent from the EEL is discharged into Peneus river.

The sampling in Psyttalia WWTP took place during a dry period, specifically in the second week of July 2025, whereas the respective sampling in Larisa WWTP took place during a wet period, specifically in the last week of November. The sampling was conducted in collaboration with the owners of WWTP facilities, specifically the Athens Water Supply and Sewerage Company (EYDAP SA) and the Municipal Water Supply & Sewerage Company of Larissa. Over the sampling campaigns, 24h flow proportional, composite influent and effluent wastewater samples were collected pre-cleaned high-density polyethylene (HDPE) 2L bottles and immediately transported at 4°C to the laboratory. The samples collected in the framework of the present study are summarized in **Table 1**.

Table 1: List of samples, analyzed in the framework of the present study.

Code	*Type of sample*	*Date of sampling*	*Flow rate (m^3/day)*
INF-WW-1	influent wastewater (CS 1: Psyttalia)	7/7/2025	597,800
INF-WW-2		8/7/2025	602,000
INF-WW-3		9/7/2025	622,700
INF-WW-4		10/7/2025	623,300
INF-WW-5		11/7/2025	617,100
INF-WW-6		12/7/2025	606,300
INF-WW-7		13/7/2025	590,100
INF-WW-8		14/7/2025	572,500
INF-WW-9	influent wastewater (CS 2: Larisa)	21/11/2025	33,000
INF-WW-10		22/11/2025	29,000
INF-WW-11		23/11/2025	48,000
INF-WW-12		24/11/2025	46,000
INF-WW-13		25/11/2025	30,000
INF-WW-14		26/11/2025	29,000
INF-WW-15		27/11/2025	28,000
EFF-WW-1	effluent wastewater (CS 1: Psyttalia)	7/7/2025	565,081
EFF-WW-2		8/7/2025	611,339
EFF-WW-3		9/7/2025	609,364
EFF-WW-4		10/7/2025	619,675
EFF-WW-5		11/7/2025	602,318
EFF-WW-6		12/7/2025	587,846
EFF-WW-7		13/7/2025	572,963
EFF-WW-8		14/7/2025	596,089
INF-WW-9	effluent wastewater (CS 2: Larisa)	21/11/2025	33,000
INF-WW-10		22/11/2025	29,000

Code	*Type of sample*	*Date of sampling*	*Flow rate (m^3/day)*
INF-WW-11		23/11/2025	48,000
INF-WW-12		24/11/2025	46,000
INF-WW-13		25/11/2025	30,000
INF-WW-14		26/11/2025	29,000
INF-WW-15		27/11/2025	28,000

All wastewater samples were processed immediately upon arrival at the Laboratory of Analytical Chemistry (LAC). Specifically, each wastewater was filtered using glass fiber filters (pore size 0.7 μm) and acidified with a 1 M HCl solution to pH 2.5 ± 0.10 to inhibit transformations and stored in the dark at −20 °C until analysis, which was carried out within one week at most [7, 8].

4. Analytical Methodology

In the framework of the present study an integrated analytical methodology was proposed, which combines validated targeted methodologies with HRMS-based screening approaches to extend the chemicals' coverage beyond predefined analytes. This integrated approach enables the detection of routinely monitored chemicals, and previously unknown substances, therefore, providing a comprehensive platform for wastewater mass and toxic load assessment and supporting an early warning system for the identification of chemicals which may pose risks to environmental and human health. It is important to highlight that although there is no analytical platform that can eliminate bias when combining data from multiple methodologies, the adopted approach minimized the systematic bias arising from extraction efficiency and matrix effect, using a thorough Quality Assurance - Quality Control approach.

In the framework of this study, three previously validated, highly sensitive, targeted methodologies were carried out for the sensitive detection of 250 pharmaceuticals, drugs of abuse, and lifestyle chemicals, 44 per- and polyfluoroalkyl substances (PFAS) and 26 tire additive chemicals (TACs) using liquid chromatography coupled to tandem mass spectrometry (triple quadrupole). The estimation of uncertainty was carried out based on the guidelines of Eurachem (Quantifying Uncertainty in Analytical Measurement, Guide CG 4, 3rd Edition) and Nordtest (Handbook for calculation of measurement uncertainty in environmental laboratories, NT TR 537, 4th Edition). Specifically, the combined uncertainty for each substance included in each target methodology was estimated using the equation: $u_c^2 = u_{bias}^2 + RSD^2 + U_{conc}^2$. The parameter u_{bias} expresses the uncertainty arising from the %Recovery, RSD represents the intermediate precision and U_{conc} arising from the uncertainty of the standard addition curve, which incorporates the most common uncertainty parameters (sample preparation, instrumental analysis). Since accredited instrumentation (e.g. mechanical pipettes) was used during sample preparation, the uncertainty arising from the use of such laboratory tools was less than the 1/3 of the combined uncertainty and, therefore, was not included in the estimation of the combined uncertainty. The maximum criterion for reliability of measurement uncertainty was 50% for the targeted methodologies according to the SANTE guideline requirements (11312/2021), therefore, the maximum estimated combined uncertainty per methodology is reported, which serves as a worst-case scenario.

4.1 Determination of pharmaceuticals and drugs of abuse using LC-MS/MS

4.1.1 Sample preparation

For the sample preparation and instrumental analysis of 250 pharmaceuticals, drugs of abuse, and their M&TPs, a previously validated [9], slightly modified analytical methodology was followed. Briefly, 50 mL of each filtrated and acidified wastewater sample was further treated. Each sample was spiked with a mixture of 35 isotopically-labelled internal standards prior to extraction. Specifically, the analytes' extraction and preconcentration were achieved with solid phase extraction (SPE) using Oasis HLB (6 cc vac cartridge, 200 mg) cartridges (Waters). The cartridges

were conditioned with 3 mL of methanol and 3 mL of milli-Q water. Following samples' loading, vacuum was applied to the SPE cartridges for 1 hour in order to dry each cartridge. The analytes' elution from the SPE cartridge was conducted using 6mL of methanol. The extracts were evaporated till dryness using a gentle stream of nitrogen at 40◦C. The samples, then, were reconstituted using 250μL of acetonitrile: milli-Q water both containing 0.1% formic acid (50/50, v/v) and were filtered through 0.45 μm RC filters in 2mL glass vials.

4.1.2 Instrumental analysis

The final extracts were analyzed using an Exion UPLC system coupled to a triple quadrupole-linear ion trap (QTRAP) mass spectrometer 6500+ (AB SCIEX, Canada) (**Figure 2**). Reversed-phase liquid chromatography using an Atlantis T3 column (100Å, 3 μm, 3 mm X 100 mm) and a gradient elution program was applied for the separation of the analytes. The mobile phases consisted of acetonitrile containing 0.1% formic acid (A) and milli-Q water containing 0.1% formic acid (B), and the injection volume was 10μL. The gradient elution program is presented in **Table 2**.

Figure 2: The mass spectrometer used for the determination of 250 pharmaceuticals, drugs of abuse, their M&TPs.

A Turbo-V ion-spray source (electrospray ionization: ESI) was used, which was operating in positive and negative ionization modes. The operating conditions were the following: Ion spray voltage: 4500 V; Source temperature: 550 °C; Curtain gas: 20 psi; Ion Source Gas 1 and 2 (GS1): 55 psi. Data acquisition was performed in switching mode using scheduled Multiple Reaction Monitoring (MRM) with a detection window equal to 180sec. Instrument control and data processing were performed using AB SCIEX Analyst software version 1.7. The determination of pharmaceuticals, drugs of abuse, their M&TPs was based on strict identification criteria; retention time shift (± 0.2 min) and ion ratio of each analyte compared to the standard solution (±30%). The method is fully validated, internal and external Quality Control procedures are in place and it is under accreditation by ISO17025. The combined uncertainty of the methodology was calculated to be equal to 25%.

Table 2: The gradient elution program, which was used for the chromatographic separation of pharmaceuticals, drugs of abuse, M&TPs.

Time (min)	*Flow rate (mL min^{-1})*	*Organic solvent %*	*Aqueous solvent %*
0.00	0.400	3.0	97.0
1.00	0.400	3.0	97.0
1.10	0.400	85.0	15.0
9.60	0.400	75.0	25.0
9.70	0.400	95.0	5.0
12.0	0.400	95.0	5.0
12.10	0.400	3.0	97.0
14.0	0.400	3.0	97.0

4.2 Determination of PFAS using LC-MS/MS

4.2.1 Sample preparation

The sample preparation workflow, which was followed for the determination of PFAS, was the same as the sample preparation procedure followed for the generic extraction of emerging contaminants (see below, paragraph 4.4.1). Briefly, the analytes' extraction and pre-concentration were carried out using layered 'mixed bed' SPE cartridges consisting of Oasis HLB and a mixture of Strata-X-AW, Strata-X-CW, and Isolute ENV+. The use of layered 'mixed bed' SPE cartridges for the determination of PFAS has already been reported in the literature [10]. Each sample was spiked with a mixture of 3 isotopically-labelled internal standards before extraction.

4.2.2 Instrumental analysis

Quantitative and qualitative analysis of the 44 PFAS in wastewater extracts was based on a previously published instrumental method [11]. Briefly, PFAS analysis was carried out in an Exion UPLC system coupled to a QTRAP mass spectrometer 6500+ (AB SCIEX, Canada). Chromatographic separation of PFAS was achieved using an Xterra MS C_{18} (2.1 x 100 mm, 3.5 μm) analytical column interfaced with the pre-column Security Guard cartridge kit (AQ C_{18} 4.0 x 3.0 mm, Phenomenex). In addition, an XBridge C_{18} (2.1 x 50 mm, 2.5 μm) delay column was used between the LC pumps and the autosampler to distinguish PFAS originated from the UPLC system and autosampler from PFAS occurred in the samples. The flow rate was set at 0.3 mL/min using methanol (A) as the organic phase and milli-Q water containing 5 mM ammonium acetate and 5 mM 1-methylpiperidine (B) as the aqueous phase. PFAS elution was based on a gradient program, with 10 % (A) for 3 min, ramping to 40 % (A) at 3.1 min, ramping to 90 % (A) at 26 min, held isocratically to 28 min and ramping back to 10 % (A) at 29 min and held until 32 min for re-equilibration.

A Turbo-V ion-spray source (ESI) was used, which was operating in negative ionization mode. The operating conditions were the following: Ion Spray Voltage, - 4500 V; Curtain Gas (CUR), 40 psi; Ion Source Gas 1 and 2, 35 psi; Source temperature 250 °C. Data acquisition was performed using Multiple Reaction Monitoring (MRM). Instrument control and data processing were performed using AB SCIEX Analyst software version 1.7. PFAS determination was based on strict identification criteria; retention time shift (± 0.2 min) and ion ratio of each analyte compared to the standard solution (±30%) in relation to a standard solution mix according to the Guidance Document of PFAS produced by EURL-POPs version 2.0. The method is fully validated, internal and external Quality Control procedures are in place, and it is accredited by ISO17025. The combined uncertainty of the methodology was calculated to be equal to 28%.

4.3 Determination of Tire Additive Chemicals using LC-MS/MS

4.3.1 Sample preparation

The sample preparation workflow, which was followed for the determination of Tire Additive Chemicals, was the same as the sample preparation procedure followed for the generic extraction of emerging contaminants (see below, paragraph 4.5.1). Briefly, the analytes' extraction and pre-concentration were carried out using layered 'mixed bed' SPE cartridges consisting of Oasis HLB and a mixture of Strata-X-AW, Strata-X-CW, and Isolute ENV+. Each sample was spiked with a mixture of 1 isotopically-labelled internal standard before extraction.

4.3.2 Instrumental analysis

Quantitative and qualitative analysis of the 15 TACs in wastewater extracts was carried out in an Exion UPLC system coupled to a QTRAP mass spectrometer 6500+ (AB SCIEX, Canada). Chromatographic separation of TACs was achieved using an Acquity UPLC BEH C_{18} Column (2.1 mm X 50 mm, 1.7 µm) analytical column. The flow rate was set at 0.3 mL/min using methanol (A) as the organic phase and milli-Q water (B) as the aqueous phase both containing 0.1% formic acid. TACs elution was based on a gradient program, with 5 % (A) ramping to 30% (A) at 2 min, ramping to 30% (A) at 2 min, ramping to 100% (A) at 4 min, held isocratically to 7 min, ramping back to 5 % (A) at 7.10 min and held until 10 min for re-equilibration. The injection volume was 5 µL and the oven temperature was 40°C.

A Turbo-V ion-spray source (ESI) was used, which was operating in positive ionization mode. The operating conditions were the following: Ion Spray Voltage, 5500 V; Curtain Gas (CUR), 40 psi; Ion Source Gas 1 and 2, 35 psi; Source temperature 350 °C. Data acquisition was performed using Multiple Reaction Monitoring (MRM). Instrument control and data processing were performed using AB SCIEX Analyst software version 1.7. TACs determination was based on strict identification criteria; retention time shift (± 0.2 min) and ion ratio of each analyte compared to the standard solution (±30%). The method is fully validated, and internal Quality Control procedures are in place. The combined uncertainty of the methodology was calculated to be equal to 44%.

4.4 Elemental analysis using ICP-MS

4.4.1 Sample preparation

Before elemental analysis using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), 10 mL of each wastewater sample was filtered using a 0.45 μm RC-45/25 filter. Afterwards, 200 μL of HNO_3 and 50 μL of HCl were added prior instrumental analysis.

4.4.2 Instrumental analysis

The elemental analysis was conducted by ICP-MS with a single quadrupole, and a collision/reaction cell. The ICP-MS model used was the Agilent 7900 (Agilent Technologies, Santa Clara, California, USA). During sample introduction, high matrix introduction (HMI) with a factor of 25 was applied to reduce the matrix effect. Analysis was performed using a collision/reaction cell containing helium and hydrogen with respective gas flow rates of 5 mL/min. The method is fully validated, internal and external Quality Control procedures are in place, and it is accredited by ISO17025. The combined uncertainty of the methodology was calculated to be equal to 10%.

4.5 Analysis of emerging contaminants using LC-TIMS-QTOF MS

4.5.1 Sample preparation

For the sample preparation of the wastewater samples, a previously published, slightly modified generic sample preparation protocol was carried out [2]. The applied analytical methodology aimed to enrich the final extracts with emerging contaminants with different physicochemical properties. Briefly, 100mL of each filtrated and acidified wastewater sample was spiked with a mix of Internal Standards, including representative isotopically labelled compounds of the database, used for wide-scope target screening. Analytes' extraction and pre-concentration were carried out using Solid Phase Extraction (SPE). Layered 'mixed bed' cartridges consisting of Oasis HLB (200 mg) and a mixture of Strata-X-AW (weak anion exchanger), Strata-X-CW (weak cation exchanger), and Isolute ENV+ (300 mg of total mixture) were used. The conditioning of the cartridges was performed with 6 mL of methanol and 6 mL of Milli-Q water. After loading the samples, the cartridges were dried by passing air through the cartridges for 0.5 to 1 h. The elution of the analytes from the adsorbent material was performed by a basic solution [6 mL of ethyl acetate/methanol (50/50 v/v) containing 2% ammonia (v/v)], followed by an acidic solution [4mL of ethyl acetate/methanol (50/50, v/v) containing 1.7% formic acid (v/v)]. The extract was evaporated to dryness under a gentle nitrogen stream at 45 °C and reconstituted to a final volume of 200 μL using a mixture of methanol and milli-Q water (50/50, v/v). The extract was filtered directly into a 2 mL vial (with an insert in it) using a syringe fitted with a 0.22 μm RC membrane filter and was ready for LC-HRMS analysis.

4.5.2 Instrumental analysis

The analysis of the final extracts was conducted using an ultra-high performance liquid chromatography (Elute LC series, Bruker Daltonics), coupled to a hybrid trapped ion mobility spectrometer quadrupole time of flight (TIMS-QTOF MS) mass spectrometer (timsTOF Pro 2, Bruker Daltonics), with a vacuum insulated probe-heated electrospray ionization (VIP-HESI) source (**Figure 3**). The LC-TIMS-QTOF MS data were acquired using broad mass range and mobility transfer acquisition mode; a data independent [broadband collision-induced dissociation (bbCID)] and a data dependent [Parallel Accumulation Serial Fragmentation (PASEF)] acquisition mode.

Figure 3: The hybrid trapped ion mobility - high resolution mass spectrometer used for the identification of emerging contaminants.

The analytes were separated in a reversed phase chromatographic column [Intensity Solo 1.8 C_{18}-2 (100 mm × 2.1 mm, 1.8 µm, Bruker Daltonics)], thermally stabilized at 40ºC. In positive ionization mode, the mobile phases consisted of (A) milli-Q water/methanol (99/1, v/v) containing 5 mM ammonium formate and 0.01% formic acid. In negative ionization mode, the mobile phases consisted of (A) milli-Q water/methanol (99/1, v/v) containing 5 mM ammonium acetate. The injection volume was 1 µL and 3 µL in positive and negative ionization modes, respectively. The gradient elution conditions are presented in **Table 3**:

Table 3: The gradient elution program, which was used for the chromatographic separation of emerging contaminants.

Time (min)	*Flow rate (mL min^{-1})*	*Aqueous solvent %*	*Organic solvent %*
0	0.200	96.0	4.0
0.1	0.200	96.0	4.0
1	0.200	81.7	18.3
2.5	0.223	50.0	50.0
14	0.400	0.1	99.9
16	0.480	0.1	99.9
16.1	0.480	96.0	4.0
19	0.480	96.0	4.0
19.1	0.200	96.0	4.0
20	0.200	96.0	4.0

The VIP-HESI source of the timsTOF Pro 2 operated using the following conditions: end plate offset 500 V; capillary voltage 2500 V; nebulizer pressure 3.0 bar; dry gas flow 10.0 L/min; dry gas temperature 220 °C; sheath gas temperature 450 °C; sheath gas flow 4.0 L/min. The LC-TIMS-QTOF MS data were acquired using broad mass range and mobility transfer acquisition mode; a data independent (bbCID) and a data dependent (PASEF) acquisition mode. Specifically, the mass to charge ratio range was 20-1300 Da, the inversed reduced ion mobility value range was 0.10-1.50 V·s/cm^2, the ramp time was 150ms, and the applied collision energies were 10.0 eV for MS and 24.0-36.0 eV for MS/MS.

External calibration was performed just before analysis with a mixture of 10 mM of sodium formate (mass calibrant) and Agilent ESI-L Low Tuning Mix (mobility calibrant) (the ratio mass: mobility calibrant is 1:3) in positive ionization mode and with a mixture of 10 mM of sodium formate (mass calibrant) and a mixture of Agilent ESI-L Low Tuning Mix and APCI/APPI Tuning Mix (1:1) (mobility calibrant) (the ratio mass: mobility calibrant is 1:3) in negative ionization mode. Also, internal calibration was performed by each calibrant injection at the beginning of each chromatogram (1st segment, 0.1−0.25 min).

4.5.3 Post-acquisition data treatment

4.5.3.1 Wide-scope target analysis

The acquired HRMS data were processed using a comprehensive, wide-scope target screening approach using an in-house database of more than 2,500 emerging contaminants [12, 13]. The post-acquisition data treatment workflow was implemented using Bruker TASQ 2025. The identification of the detected analytes based on strict identification criteria: mass accuracy (±2mDa), retention time shift (±0.2min), isotopic pattern matching, presence of qualifier ions (MS1 ions: adduct ions, in-source fragments, and MS2 fragment ions), and %ΔCCS values (±2%).

The method is fully validated, and internal Quality Control procedures are in place. The combined uncertainty of the methodology was calculated to be equal to 44%.

4.5.3.1 Suspect screening

HRMS-based suspect screening is increasingly recognized as a justified and scientifically robust approach for regulatory applications, particularly in the monitoring of emerging contaminants in wastewater [1, 14, 15]. Unlike conventional target monitoring, which is restricted to a limited number of preselected and regulated substances, HRMS-based suspect screening enables systematic monitoring of hundreds to thousands of relevant chemicals, many of which lack reference standards or regulatory thresholds and, thus, are overlooked from the conventional monitoring programs [16]. Although the resulting data are mainly semi-quantitative, they provide comprehensive and reliable evidence on the presence, distribution, and estimated concentration of organic pollutants in complex environmental matrices, such as wastewater. This information is sufficient to support regulatory needs such as early warning, exposure screening, and prioritization of substances for further assessment. Suspect screening aligns well with the tiered structure of regulatory frameworks, where it can serve as a first screening step to justify and trigger targeted monitoring, risk assessment, or mitigation measures. When implemented under harmonized protocols, minimum quality requirements, and transparent confidence levels for identification, HRMS-based suspect screening produces reproducible and defensible data [17]. Moreover, the ability to archive the acquired HRMS data and perform retrospective analyses as new substances of concern or updated suspect lists emerge further strengthens the regulatory value of this approach, as it allows authorities to respond to emerging risks without resampling. At the European level, HRMS-based suspect screening is now a well-established and widely applied analytical approach, having been implemented in numerous research initiatives, collaborative projects, and routine environmental monitoring programs to address the growing challenge of emerging pollutants in wastewater and aquatic environments. Therefore, its integration into regulatory monitoring is scientifically justified, as it enhances the chemical coverage, efficiency, and responsiveness of environmental legislation such as the Water Framework Directive, the Marine Strategy Framework Directive, and REACH, ultimately supporting evidence-based and forward-looking policy decisions.

Suspect screening was performed using the 3 suspect lists included in NORMAN Substance List Exchange platform [18–20]. The suspect lists used in the present study included 559 surfactants, 1,987 cosmetics, and 629 industrial and plastic-related chemicals, retrieved from NORMAN Suspect List Exchange (https://www.norman-network.com/nds/susdat/) by applying the respective chemical class criteria [Surfactants (SRF), Personal Care Products (PCP), Plastic Additives (PLAST) and Industrial Chemicals (IND)]. The suspect screening was carried out in Bruker MetaboScape 2025, which enables the identification of "known unknown chemicals" in environmental samples through suspect and non-target screening. Briefly, internal calibration was applied in the mass and mobility PASEF data to reduce mass and mobility deviation across the samples. The Time-aligned Region complete Extraction (T-ReX 4D) peak-picking algorithm was

used to generate a feature table from the acquired PASEF data. Each feature included retention time, m/z, CCS value, and peak area information. If MS/MS data are available, the highest-intensity MS/MS spectrum is automatically assigned to each feature. Novel chemoinformatic tools were utilized, aiming to predict analytical information (retention time, collision cross section - CCS-values) from the respective structural information (SMILES, InChI), included in each suspect list [21]. Moreover, *in-silico* fragmentation prediction was utilized by MetFrag [22], which was incorporated inside MetaboScape. MetFrag induced in-silico fragmentation of the compound structures with subsequent MS/MS fragment peak matching with the predicted ones. Each annotated feature was thoroughly evaluated based on the mass error of the precursor ion (±5mDa), predicted retention time shift (±0.5min), isotopic pattern matching, *in-silico* MS/MS spectrum, and predicted CCS value matching (±5%). An advanced quantitative structure–property relationship (QSPR)-based chemoinformatic model was used to predict the ionization efficiency of the annotated compounds, which was used for their semi-quantification [23, 24]. Semi-quantification is commonly applied in HRMS-based suspect screening to provide an estimation of pollutant levels in the absence of reference standards, allowing comparison of relative concentrations across samples and supporting prioritization and regulatory decision-making despite inherent uncertainties [25]. LogIE values from ESI source, which were available in previous publication were calibrated by the VIP-HESI source and used for the subsequent semi-quantitative analysis. Semi-quantification is commonly applied in HRMS-based suspect screening to provide an estimation of environmental pollutant levels in the absence of reference standards, allowing comparison of relative concentrations across samples and supporting prioritization and regulatory decision-making despite inherent uncertainties. The method was fully validated, and external Quality Control procedures (a collaborative trial) were performed to estimate the combined uncertainty of the methodology. Combined uncertainty of this methodology was calculated at 75%.

4.6 Quality Assurance – Quality Control

A thorough Quality Assurance – Quality Control (QA-QC) approach was followed during the sample preparation and the instrumental analysis. The efficiency of the extraction was monitored using representative isotopically labelled internal standards (IS). Specifically, the internal standards' mixture used in the present study included the following representative isotopic labelled compounds of the studied emerging contaminants: Flunixin-D_3, BPA-D_{16}, Ranitidine-D_6, Diuron-D_6, Fenbendazole-D_3, Meloxicam-D_3, Atrazine-D_5, Mefenamic acid-D_3, Diazepam-D_5, Sulfamerazine-D_4, Sulfadiazine-D4, Tebuconazole-D_9, Caffeine-D_9, Carbendazim-D_4, Metformin-D_6, Cetirizine-D_8, Atenolol-D_7, Imidacloprid-D_4, Irbesartan-D_4, Sucralose-D_6, Valsartan-D_9, Venlafaxine-D_6, Amisulpride-D_5, Amitryptiline-D_3, Atorvastatin-D_5, Azithromycin-D_3, Citalopram-D_4, Codeine-D_6, Diclofenac-D_6, Erythromycin-D_6, Fluconazole-D_4, Fluoxetine-D_5, Ibuprofen-D_3, Levofloxacin-D_3, Naproxen-D_3, Propranolol-D_7, Tramadol-D_6, M_8PFOS, M_8PFOA and M_8PFOSA.

As described in detail by previous publications [26], QC was underpinned by the simultaneous processing and evaluation of procedural blanks, standards, including representative compounds of

the analytes included in the target lists and spiked samples during the analysis of all samples. The reported concentration levels were corrected using procedural blanks, aiming to minimize the false positive concentrations attributed to cross-contamination during sample preparation and instrumental analysis. Two mixes (for positive and negative ionization mode, respectively), each containing 18 known substances (Retention Time Indices -RTI- calibrant compounds), were injected in the sequence for the assessment of retention time shift and for additional data treatment of the chromatograms following suspect and non-target screening workflows. A QC sample was analyzed every ten injections to monitor analytical performance and secure enhanced mass spectrometer sensitivity.

The quantification of the detected compounds was performed using the standard addition method and representative structurally related isotope-labeled compounds. A sample matrix with the lowest detected compound number and concentration levels was used for the quantification experiments, as contaminant-free wastewater is not commercially available for chemical analysis purposes. Linearity was calculated and evaluated, whereas % Recoveries and % Factors of Matrix Effects were calculated in different concentration levels using standard solutions, spiked and matrix-matched samples (analytes were spiked before extraction and before instrumental analysis, respectively). Method limits of detection (LODs) were calculated from standard addition curves (using relative peak areas of spiked samples) with the following equation: $LOD = 3.3*(S_b/S)$, where S is the slope of the calibration curve, and S_b is the standard deviation of the response. The contaminants that were detected in traces below the limit of quantification (LOQ) were reported as <LOQ. For the statistical treatment of the results, the substitution of <LOQ with LOQ/2 and <LOD with LOD/2 has been performed, as indicated by Directive 2009/90/EC.

Regarding elemental analysis, a 7-point calibration curve was prepared for the quantification of the samples, covering a concentration range of 0.1–1000 μg/L based on the expected concentration of each metal in the sample solution. The evaluation of the system's suitability was performed using two standard solutions from a different provider, with concentrations of 2 μg/L and 20 μg/L for all metals except Hg, for which the concentrations were 0.2 μg/L and 2 μg/L for the low and high concentration level, respectively. The overall method's quality control (QC) sample was prepared by spiking a sample at the central point of the calibration curve.

4.7 Ecotoxicological Risk Assessment

To conduct a thorough ecotoxicological risk assessment, a database (https://pnec.chem.uoa.gr) containing **28,734 LC_{50} values for 30 freshwater species**, covering the three base-groups of fish, crustaceans and algae, was created to derive **Provisional No-Effect Concentrations (PNEC)**, using *an AF of 1000 in case of acute toxicity data while an AF of 10.0 was used for chronic PNECs*. Data was retrieved from open-source repositories from indicative research centers and legislative parties, both European and international (such as NITE, US EPA) and 200 peer-reviewed research papers. For identified chemicals lacking experimental ecotoxicity data, experimental-based lowest PNECs were retrieved from the ecotox module of the NORMAN Database System. Moreover,

where no experimental toxicity data was found, a read-across approach was used to find ecotoxicity data for similar chemicals.

Structural representation information (InChIKey, SMILES) was retrieved for compounds with no available experimental ecotoxicity data. A Tanimoto similarity tool was developed that allowed for the selection of similar compounds in the experimental dataset, based on the structural information, from which ecotoxicological values were retrieved. The Tanimoto similarity approach is widely regarded as an appropriate tool for fingerprint-based similarity calculations [27], due to its strong theoretical compatibility with binary molecular representations and its demonstrated empirical performance. Molecular fingerprints encode the presence or absence of structural features as sparse binary vectors, and the Tanimoto coefficient directly quantifies similarity as the ratio of shared features to the total number of features present in each molecule. This formulation minimizes bias arising from differences in fingerprint length or feature density and provides stable similarity rankings across diverse chemical datasets. A four-level system was adopted from the literature [27] to characterize the confidence of the read-across assignments based on the degree of structural similarity. Specifically, as proposed by Mellon et al. (2019), Tanimoto coefficients of ≥0.75 can be considered indicative of high structural similarity (level 1). Lower coefficients can be classified into three additional levels [level 2 (values between 0.5 and 0.749), level 3 (values between 0.3 and 0.499) and level 4 (values below 0.299)], representing progressively lower degrees of structural similarity. These Tanimoto-based categories should be regarded as indicative rather than as universally established threshold for assigned toxicity value validity.

The subsequently derived PNEC values were used for the %toxic load calculation per chemical class using equation 3. The equation yields the daily toxic load per chemical class in which the values are obtained by simply summing compound-level values. The % contribution of each class is then derived by simply dividing the class-specific toxic load by the total toxic load.

$$\text{Toxic load}\left(\frac{\text{TU}\cdot\text{PE}}{\text{day}}\right)=\sum\left(\frac{\text{MEC}}{\text{DF}\cdot\text{PNEC}}\right)\cdot\frac{\text{flow}}{\text{per capita WF}} \quad [\mathbf{Eq.\,3}]$$

RQ: Risk Quotient

PE: population equivalent value

MEC: measured environmental concentration

DF: dilution factor

WF: wastewater factor

Effluent flows per day, dilution factors to receiving water (150 for the Psyttalia WWTP; 110 for the Larissa WWTP) and per capita wastewater factor [200 L (PE day)$^{-1}$ for the Psyttalia WWTP;

120 L (PE day)$^{-1}$ for the Larissa WWTP] were kindly provided from the management authorities of both WWTPs, namely EYDAP SA and DEYAL, respectively.

Class-level toxic loads were calculated by summing compound-level values within each chemical class. Chemical classes and individual compounds were then ranked by their percentage contribution to the cumulative toxic load of the effluent. This approach was preferred over simply determining the share of compounds exceeding their PNEC (i.e. frequency of exceedance), since it captures the cumulative pressure of the chemical mixture, including compounds whose individual risk quotient is below 1, but which collectively contribute to mixture toxicity.

5. Results

The results reported herein are based on data and analyses that have been published in a scientific article [5]. This technical report represents an extended and detailed version of the published manuscript, providing additional information, data interpretation, and technical details to support the reported findings.

5.1 Results from targeted analyses in Psyttalia WWTP

The comprehensive targeted analyses by LC-MS/MS and LC-HRMS, conducted at the Laboratory of Analytical Chemistry, revealed that *341 compounds*, grouped into different chemical classes based on their main use, were detected and quantified in influent and effluent wastewater samples.

339 chemicals were determined in **influent** wastewater samples. The dominant chemical class of the detected compounds was pharmaceuticals, M&TPs (49%, n=167), followed by plant protection products (11%, n=36) and industrial chemicals (9.1%, n=31). The rest compounds, classified as: drugs of abuse (6.7%, n=23), TACs and PFAS (4.7% each, n=16), surfactants (4.1%, n=14), personal care products (3.8%, n=13), coffee and tobacco related chemicals (2.9%, n=10), veterinary drugs (2.7%, n=9) and artificial sweeteners (1.2%, n=4).

The highest proportion of chemicals belongs to the pharmaceuticals due to the specific target list that contains >900 of those chemicals, plus the fact of their diversity and their widespread use throughout society. Moreover, numerous metabolites and transformation products were determined due to different metabolic pathways, as well as different degradation patterns.

Similarly, in **effluent** wastewater samples, 300 chemicals were determined. The subclass order followed the same pattern as influent wastewater. Specifically, the dominant chemical class of the detected compounds was pharmaceuticals, M&TPs (50%, n=151), followed by plant protection products (11%, n=33) and industrial chemicals (8.6%, n=26). The rest compounds, classified as: drugs of abuse and TACs (5.3% each, n=16), PFAS and surfactants (4.6% each, n=14), personal care products (3.3%, n=10), coffee and tobacco related chemicals (0.3%, n=9), veterinary drugs (0.26%, n=8) and artificial sweeteners (0.01%, n=3).

Measured concentration levels for all detected chemicals were normalized with daily WWTP flow rates and calculated as mass loads (g/day). The mass loads in influent wastewater samples are depicted in the following bar chart (**Figure 4**) and in **Table 4**.

Table 4: Median aggregated mass load (g/d) of each chemical class in analyzed *influent* wastewater samples, collected from KELPS WWTP and its' contribution in the total mass load.

Class	*Median ∑ mass load (g/d)*	*percentage*
Pharmaceuticals, M&TPs	86,633	37%
Coffee and Tobacco related compounds	68,738	29%
Industrial Chemicals	27,595	12%
Artificial Sweeteners	17,156	7.2%
Surfactants	15,316	6.5%
Drugs of Abuse, M&TPs	6,973	2.9%
Plant Protection Products, M&TPs	5,800	2.5%
Personal Care Products, M&TPs	4,776	2.0%
Veterinary Drugs, M&TPs	2,343	0.99%
Tire Additive Chemicals	1,007	0.43%
Per- and Polyfluoroalkyl Substances	318	0.13%

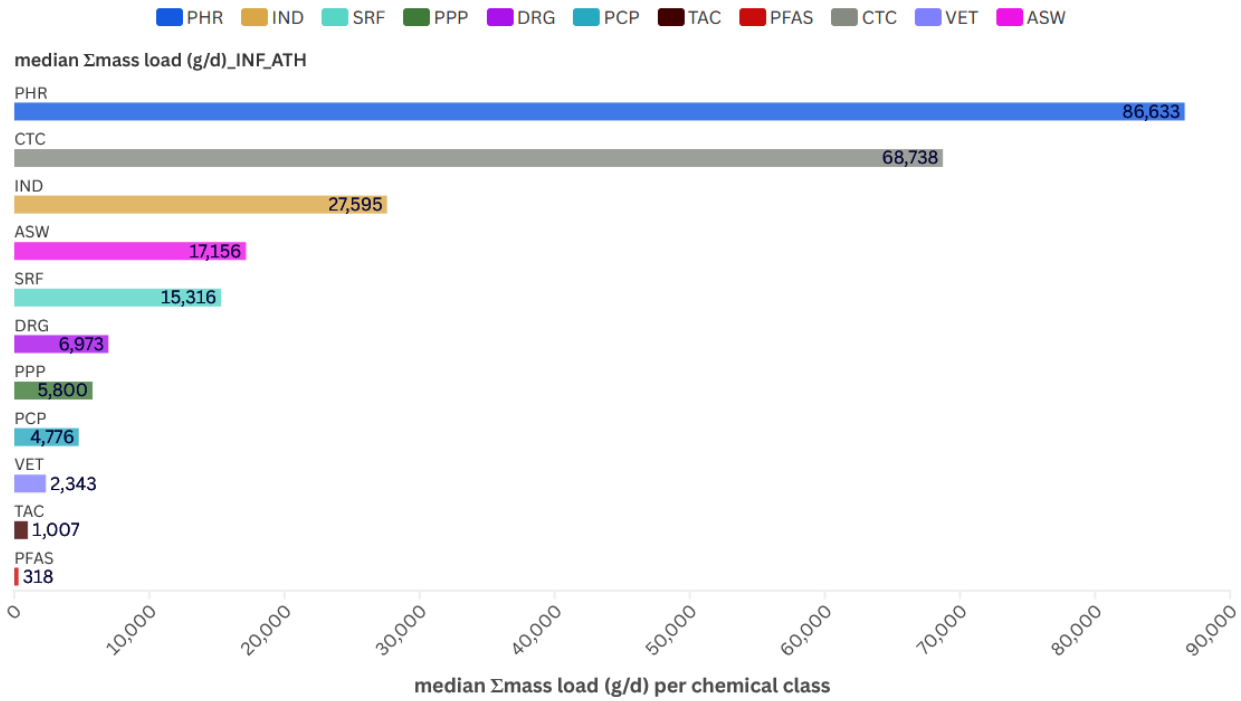

Figure 4: Median aggregated mass loads of determined chemicals through targeted analysis in the influent wastewater collected from KELPS WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

Specifically, percentages of mass loads per chemical class in influent wastewater show that pharmaceuticals do not exceed 40% of the total chemical load on average. In contrast, chemical classes with a smaller number of chemicals, such as coffee and tobacco related compounds (29%), industrial chemicals (12%) and surfactants (6.5%) cover an almost equal percentage of the total chemical burden. It is also noted that the toxicity, endocrine-disrupting properties, as well as persistence and bioaccumulation potential of these chemical groups have not yet been extensively studied.

As discussed above, the results of this monitoring study clearly demonstrate that a substantial proportion of the chemicals detected in wastewater are pharmaceutical compounds. As noted by Kummerer et al. (2008), pharmaceutical residues in urban wastewater primarily originate from human consumption followed by excretion in the sewer system [28]. Detailed examination of the detected compounds shows that the majority are metabolites and transformation products rather than parent pharmaceuticals. This observation strongly supports the conclusion that the presence of these substances in wastewater results mainly from human consumption rather than from direct disposal. Within the framework of wastewater-based epidemiology, *the ratio between parent drug and metabolite (P:M) is a widely accepted indicator for distinguishing consumption patterns from disposal events* [29]. The findings of this study show *consistent P:M ratios across sampling periods*, indicating that the *detected quantities reflect human consumption* (**Table 5**). A representative example is losartan and its primary metabolite, losartan carboxylic acid. Pharmacokinetic data demonstrate that approximately 4% of the administered dose is excreted unchanged, while around 6% is excreted as losartan carboxylic acid. These proportions comply with the ratios observed in wastewater, where the total concentration of losartan detected is divided into 38% losartan and 62% losartan carboxylic acid, with a negligible deviation of 4%. It should be noted that P:M ratios may vary between pharmaceutical substances due to factors such as in-sewer degradation, differences in formulation, administration route, or co-consumption of other substances. Nevertheless, for a specific wastewater treatment plant, the historical average P:M ratio for each compound is expected to remain relatively stable. **Any significant deviation from this established baseline should therefore be interpreted as a potential indicator of a disposal incident. Table 5** presents the ratios for several pharmaceuticals and their metabolites included in this study, **providing further confirmation that their presence in wastewater is attributable to human consumption rather than direct disposal**.

Table 5: Parent drug to metabolites ratio for the detected pharmaceutical in KELPS WWTP.

Pharmaceutical	Extracted substances	Ratio (%)
Carbamazepine	Carbamazepine	14 ± 4
	10,11-Dihydro-10-hydroxycarbamazepine	82 ± 7
	2-Hydroxycarbamazepine	5 ± 2
Citalopram	Citalopram	89 ± 1
	Norcitalopram	6 ± 1

Pharmaceutical	Extracted substances	Ratio (%)
	3-oxocitalopram	6 ± 1
Omeprazole	Omeprazole	78 ± 5
	Omeprazole sulfone	22 ± 5
Quetiapine	Quetiapine	54± 5
	Norquetiapine	46± 5
Tramadol	Tramadol	62 ± 3
	O-Desmethylnortramadol	2 ± 1
	O-Desmethyltramadol	37± 3
Venlafaxine	Venlafaxine	49 ± 9
	O-desmethylVenlafaxine	44± 7
	N-Desmethylvenlafaxine (Norvenlafaxine)	3 ±1
	D,L-N,O-Didesmethyl venlafaxine	4 ±1
Sulfamethoxaxole	Sulfamethoxazole	47± 1
	N4-acetylsulfamethoxazole	53 ±1
Amitryptiline	Amitriptylline	93 ±2
	Nortriptyline	8 ± 2
Clopidogrel	Clopidogrel	4 ± 0,5
	Clopidogrel carboxylic acid	96 ± 0,5
Mirtazapine	Mirtazapine	46 ± 1
	8-OH-Mirtazapine	38 ± 2
	Normirtazapine	16 ± 1
Diltiazem	Diltiazem	14 ± 10
	Desacetyl diltiazem	86 ± 10
Metoprolol	Metoprolol	32 ± 9
	Metoprolol acid	68 ± 9
Metformin	Metformin	12 ± 4
	Guanylurea	88 ± 4
Losartan	Losartan	38 ± 4
	Losartan carboxylic acid	62 ± 4
Zolpidem	Zolpidem	3 ± 1
	Zolpidem carboxylic acid	97 ± 1
Sildenafil	Sildenafil	63 ± 7
	Norsildenafil	37 ± 7
Lidocaine	Lidocaine	93 ± 1
	Norlidocaine	7 ± 1

The median mass loads per chemical class in **effluent** wastewater, collected from KELPS WWTP, are summarized in **Table 6** and depicted in **Figure 5**.

Table 6: Median aggregated mass load (g/d) of each chemical class in analyzed *effluent* wastewater samples, collected from KELPS WWTP and its' contribution in the total mass load.

Class	*Median ∑ mass load (g/d)*	*percentage*
Pharmaceuticals, M&TPs	25,728	48%
Coffee and Tobacco related compounds	2,307	4.3%
Industrial Chemicals	5,700	11%
Surfactants	10,000	19%
Artificial Sweeteners	1,657	3.1%
Drugs of Abuse, M&TPs	264	0.49%
Plant Protection Products, M&TPs	2,163	4.1%
Personal Care Products, M&TPs	2,332	4.4%
Veterinary Drugs, M&TPs	1,611	3.0%
Tire Additive Chemicals	1,443	2.7%
Per- and Polyfluoroalkyl Substances	120	0.23%

Besides the total chemical load in **influent** wastewater, the **removal efficiency** of KELPS WWTP is also noteworthy. Median mass loads per chemical class in both **influent** and **effluent** wastewater samples and the **average %removal** per chemical class are summarized in **Table 7**.

Table 7: Median aggregated mass loads (g/d) and number of detected compounds through target analyses in KELPS WWTP case study. The average %removal of each chemical class is also presented on the table. The following abbreviations were used in the table: PHR=pharmaceuticals, M&TPs, CTC=coffee and tobacco related compounds, IND=industrial chemicals, SRF=surfactants, ASW=artificial sweeteners, DRG=drugs of abuse, PPP=plant protection products, PCP=personal care products, VET=veterinary drugs, TAC= tire additive chemicals, and PFAS=per-and polyfluoroalkyl substances.

Class	*Influent wastewater*		*Effluent wastewater*		*Average %Removal*
	Number of compounds	*Median ∑ mass load (g/d)*	*Number of compounds*	*Median ∑ mass load (g/d)*	
PHR	167	86,633	151	25,728	-70%
CTC	10	68,738	9	2,307	-96%
IND	31	27,595	26	5,700	-80%
ASW	4	17,156	3	1,657	-91%
SRF	14	15,316	14	10,000	-52%
DRG	23	6,973	16	264	-96%
PPP	36	5,800	33	2,163	-60%
PCP	13	4,776	10	2,332	-52%
VET	9	2,343	8	1,611	-26%
TAC	16	1,007	16	1,443	**+11%**
PFAS	16	318	14	120	-61%

As summarized in **Table 7**, pharmaceuticals exhibit notably higher removal efficiencies compared to other chemical groups, including personal care products (e.g., cosmetics, UV filters, skin cleansers and synthetic musks), surfactants (e.g., biocides, antiseptics, detergents and cleansers), veterinary medications, and TACs. The latter display a removal ratio equal to 11%, indicating their recalcitrant nature, thus no practical removal and stable overall mass load during the wastewater treatment process. TACs represent an emerging class of environmental pollutants for which scientific knowledge remains limited. Although their full toxicological profile is not yet understood, growing evidence points to their potential endocrine-disrupting properties, underscoring the need for further investigation.

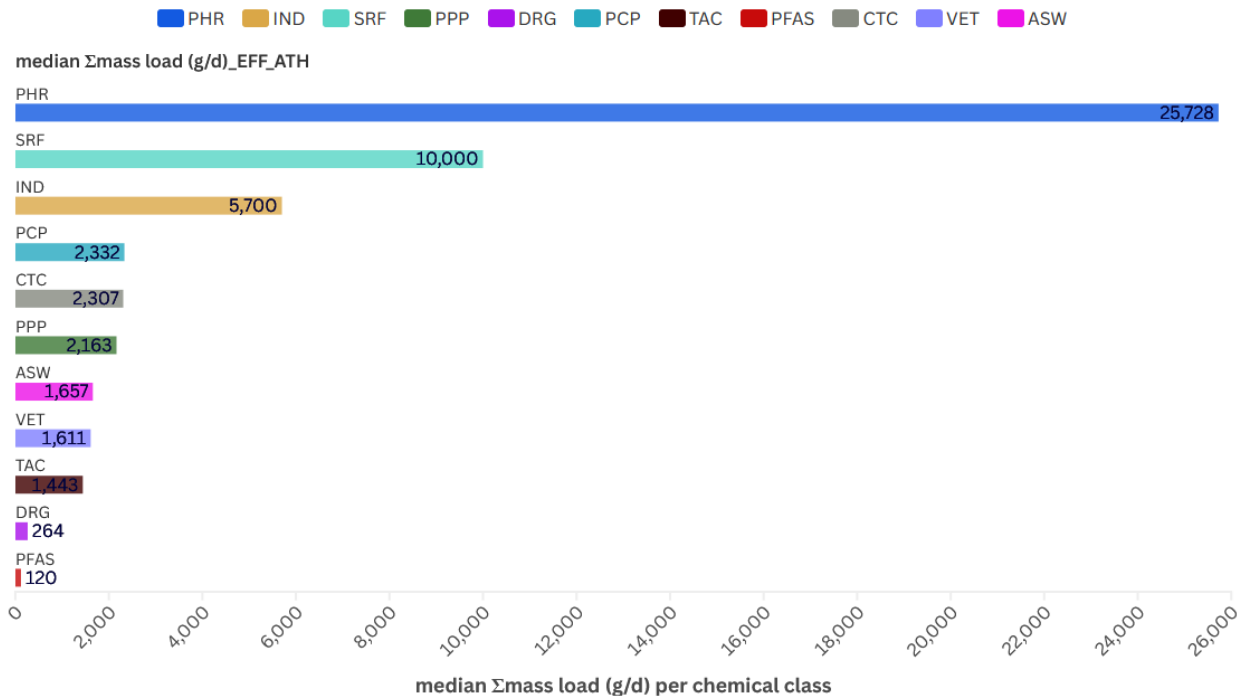

Figure 5: Median aggregated mass loads of the determined chemicals through *targeted analysis* in the *effluent* wastewater, collected from KELPS WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

5.2 Results from target analysis, suspect screening & elemental analysis in Psyttalia WWTP

A comprehensive suspect screening workflow was carried out, aiming to investigate the occurrence of important anthropogenic chemicals for which the analytical standards were not available. For that reason, extensive suspect list of cosmetics, surfactants and plastic related chemicals were used. Moreover, the presence of heavy metals was investigated in the samples aiming to unravel the complete chemical burden of wastewater samples from both organic and inorganic chemicals. Additional *76 chemicals were identified* in level 2a or 3, based on the confidence scheme proposed

by Celma et al. (2020) [30]. As depicted in the following figures, the chemical profile has been dramatically changed in terms of the number of chemicals per chemical class detected and mass loads in influent and effluent samples observed.

After incorporating tentatively identified substances *via* suspect screening the depiction of the chemical content of influent wastewater appears differently compared to the chemical profile for the targeted analyses, which indicated that there are many compounds that are "hidden" in the samples due to the unavailability of commercial analytical standards. In brief, a total of *435 compounds were identified in influent wastewater*. These belong to pharmaceuticals, M&TPs (38%, n=167), industrial chemicals (13%, n=58), surfactants (10%, n=45), plant protection products (8.3%, n=36), personal care products, including tentatively identified cosmetics (7.1%, n=31), drugs of abuse (5.3%, n=23), (heavy) metals (4.6%, n=20), TACs and PFAS (3.7% each, n=16), coffee and tobacco related compounds (2.3%, n=10), veterinary drugs (2.1%, n=9) and artificial sweeteners (0.92%, n=4). In this case, the pharmaceuticals along with their metabolites and TPs, do not exceed 40% of the total determined compounds, not even in terms of number of identified substances. It should be noted that many chemicals used as intermediates in manufacturing processes, or household cleaners, or antioxidants and stabilizers in various industrial and consumer products, and plastic related chemicals have been classified as *industrial chemicals*. The chemicals have been classified based on their main use, although they may also have other uses.

Similarly, in effluent wastewater the determined substances (n=378) belong to pharmaceuticals, M&TPs (40%, n=151), industrial chemicals (13% n=48), surfactants (9.5%, n=36), plant protection products (8.7%, n=33), personal care products (6.3%, n=24), (heavy) metals (5.3%, n=20), drugs of abuse (4.2%, n=16), TACs (4.2%, n=16), PFAS (3.7%, n=14), coffee and tobacco related compounds (2.4%, n=9), veterinary drugs (2.1%, n=8) and artificial sweeteners (0.79%, n=3).

Moreover, an entirely different profile of the influent wastewater mass load is presented in **Table 8**, when suspect screening results are incorporated. As presented in **Figure 6**, pharmaceuticals cover a mere 2% of the total chemical burden, on average, in influents. Notably, industrial chemicals, surfactants, and personal care products (including cosmetics-related chemicals) comprise 41%, 41% and 14% of the total chemical load, respectively.

Table 8: Median aggregated mass load (kg/d) and contribution of each chemical class in the total mass load in the analyzed *influent* wastewater samples, collected from KELPS WWTP.

Class	*Median ∑ mass load (kg/d)*	*percentage*
Industrial Chemicals	1,876	41%
Surfactants	1,843	41%
Personal Care Products, M&TPs	630	14%
Pharmaceuticals, M&TPs	86.6	1.9%
Coffee and Tobacco related compounds	68.7	1.5%
Artificial Sweeteners	17.2	0.38%
Drugs of Abuse, M&TPs	6.97	0.15%
Plant Protection Products, M&TPs	5.80	0.13%
Veterinary Drugs, M&TPs	2.34	0.052%
Tire Additive Chemicals	1.00	0.022%
Per- and Polyfluoroalkyl Substances	0.318	0.0070%

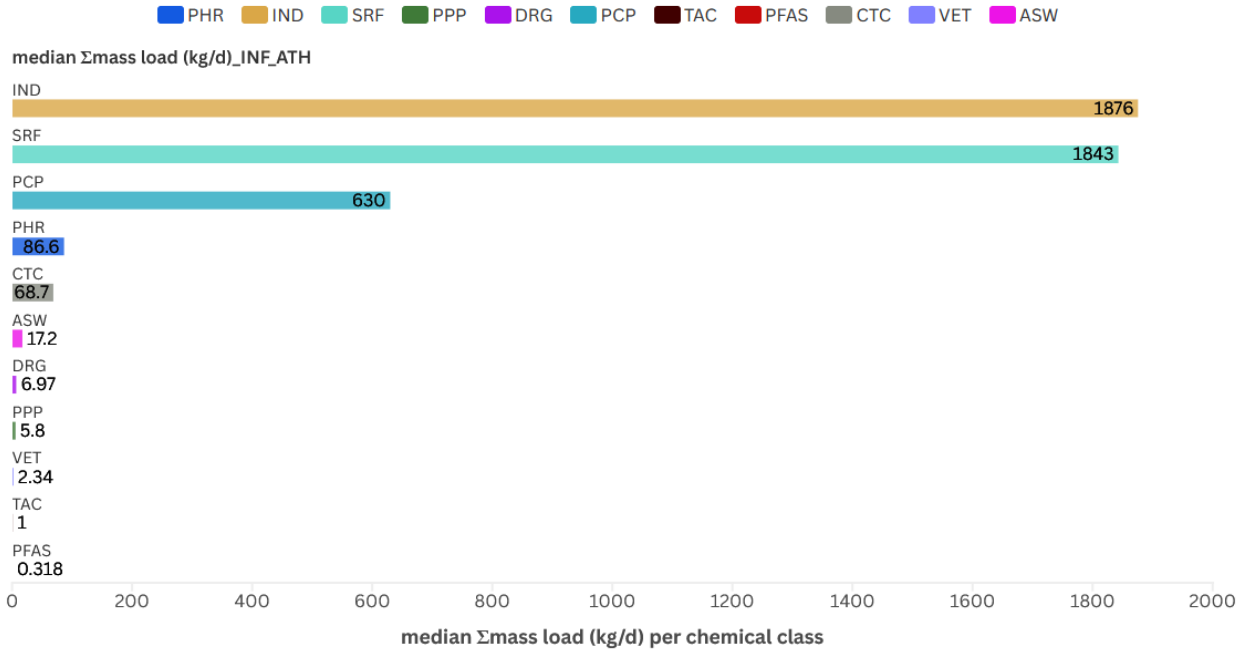

Figure 6: Median aggregated mass loads of the determined organic chemicals through target and suspect screening analysis in *influent* wastewater samples, collected from KELPS WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

The median aggregated mass loads per chemical class in *effluent* wastewater, collected from KELPS WWTP, are summarized in **Table 9** and depicted in **Figure 7**.

Table 9: Median aggregated mass load (kg/d) and contribution of each chemical class in the total mass load in the analyzed *effluent* wastewater samples, collected from KELPS WWTP.

Class	*Median ∑ mass load (kg/d)*	*percentage*
Industrial Chemicals	59.7	49%
Surfactants	13.2	11%
Personal Care Products, M&TPs	14.3	12%
Pharmaceuticals, M&TPs	25.6	21%
Coffee and Tobacco related compounds	2.29	1.87%
Artificial Sweeteners	1.65	1.35%
Drugs of Abuse, M&TPs	0.262	0.21%
Plant Protection Products, M&TPs	2.15	1.76%
Veterinary Drugs, M&TPs	1.60	1.30%
Tire Additive Chemicals	1.43	1.16%
Per- and Polyfluoroalkyl Substances	0.120	0.10%

The removal efficiency is further evaluated by the integration of HRMS-based suspect screening results (**Table 10**). Targeted and suspect-screening data indicate that pharmaceuticals exhibit approximately 0.5 orders of magnitude reduction during treatment. In contrast, PFAS, plant-protection products, and veterinary drugs show considerably lower removal efficiencies, persisting in the wastewater stream after the clean-up process. Moreover, certain chemical classes - particularly tire additive chemicals - display increased mass loads following treatment, denoting no removal during treatment. These groups include emerging contaminants, such as 6-PPD-quinone, as well as various benzothiazoles.

Table 10: Median aggregated mass loads (kg/d) and number of detected compounds through target and suspect screening analyses in KELPS WWTP case study. The average (%) removal of each chemical class is also presented on the table. The following abbreviations were used in the table: PHR=pharmaceuticals, M&TPs, CTC=coffee and tobacco related compounds, IND=industrial chemicals, SRF=surfactants, ASW=artificial sweeteners, DRG=drugs of abuse, PPP=plant protection products, PCP=personal care products (including cosmetics), VET=veterinary drugs, TAC= tire additive chemicals, and PFAS=per-and polyfluoroalkyl substances.

Class	*Influent wastewater*		*Effluent wastewater*		*Average %Removal*
	Number of compounds	*Median ∑mass load (kg/d)*	*Number of compounds*	*Median ∑ mass load (kg/d)*	
IND	58	1,876	48	59.7	-97%
SRF	45	1,843	36	13.2	-99%
PCP	31	630	24	14.3	-96%
PHR	167	86.6	151	25.6	-70%
CTC	10	68.7	9	2.29	-96%
ASW	4	17.2	3	1.65	-91%

Class	*Influent wastewater*		*Effluent wastewater*		*Average %Removal*
	Number of compounds	*Median ∑mass load (kg/d)*	*Number of compounds*	*Median ∑ mass load (kg/d)*	
DRG	23	6.97	16	0.262	-96%
PPP	36	5.80	33	2.15	-60%
VET	9	2.34	8	1.60	-26%
TAC	16	1.00	16	1.43	**+11%**
PFAS	16	0.318	14	0.120	-61%

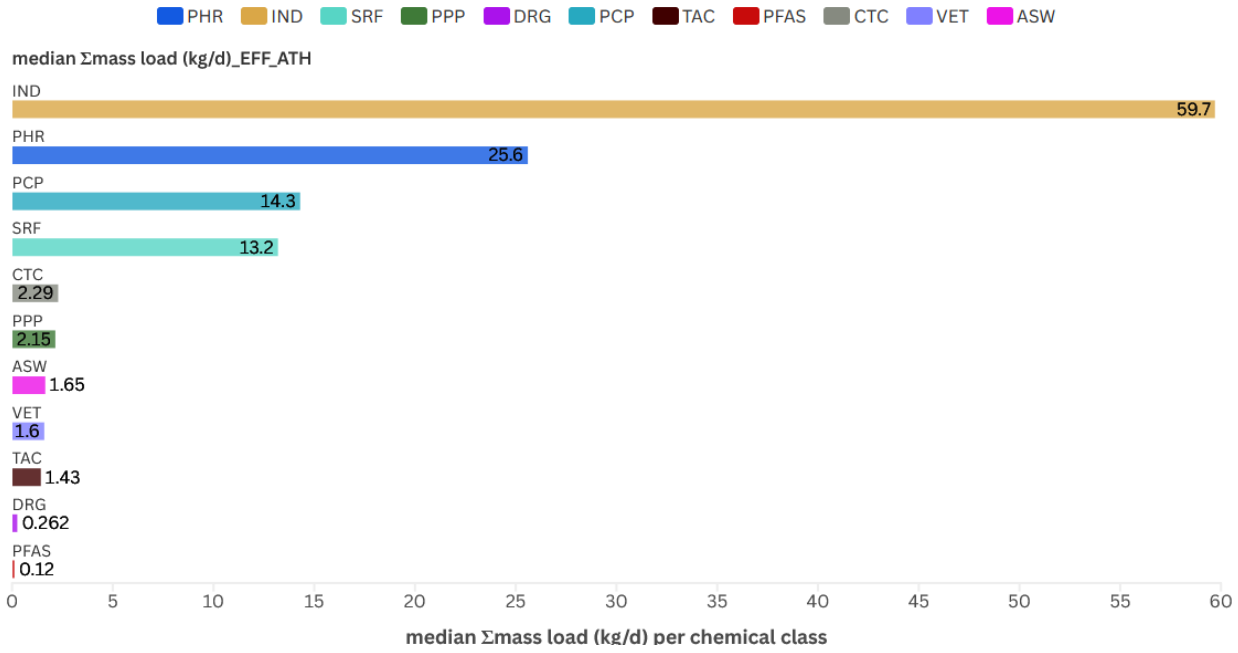

Figure 7: Median aggregated mass loads of the determined chemicals through target and suspect screening analysis in *effluent* wastewater, collected from KELPS WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

5.3 Results from targeted analyses in Larissa WWTP

The comprehensive targeted analyses by LC-MS/MS and LC-HRMS conducted at LAC revealed that *296 compounds*, grouped into different chemical classes based on their main use, were detected and quantified in influent and effluent wastewater samples.

291 chemicals were determined in **influent** wastewater samples. The dominant chemical class of the detected compounds were pharmaceuticals and M&TPs (54%, n=157), followed by plant protection products (9.6%, n=28) and industrial chemicals (8.9%, n=26). The rest of the determined compounds, classified as: drugs of abuse (6.2%, n=18), TACs (5.2%, n=15), surfactants (4.8%, n=14), coffee and tobacco related chemicals (3.4%, n=10), per- and polyfluorinated alkyl substances (2.7%, n=8), personal care products (2.4%, n=7), veterinary drugs (1.7%, n=5) and artificial sweeteners (1.0%, n=3). Again, the highest proportion of chemicals belongs to the pharmaceuticals due to the specific target list and diverse consumption by the population.

Similarly, in **effluent** wastewater samples, 281 chemicals were determined. The subclass order followed the same pattern as influent wastewater. Specifically, the dominant chemical class of the detected compounds was pharmaceuticals, M&TPs (55%, n=155), followed by plant protection products and industrial chemicals (8.9%, n=25, each). The rest compounds, classified as: TACs and surfactants (5.3%, n=15, each), drugs of abuse (5.0%, n=14), coffee and tobacco related chemicals (3.6%, n=10), PFAS (3.2%, n=9), personal care products (2.1%, n=6), veterinary drugs (1.8%, n=5) and artificial sweeteners (0.71%, n=2).

Measured concentration levels for all detected chemicals were normalized with daily WWTP flow rates and calculated as mass loads (g/day). The mass loads in **influent** wastewater samples are depicted in the following pie chart (**Figure 8**) and in **Table 11**.

Table 11: Median aggregated mass load (g/d) and contribution of each chemical class in the total mass load in analyzed *influent* wastewater samples, collected from EEL WWTP.

Class	Median ∑ mass load (g/d)	Percentage
Plant Protection Products, M&TPs	14,621	50%
Coffee and Tobacco related compounds	6,803	23%
Pharmaceuticals, M&TPs	5,827	20%
Industrial Chemicals	764	2.6%
Surfactants	546	1.9%
Artificial Sweeteners	473	1.6%
Drugs of Abuse, M&TPs	184	0.63%
Personal Care Products, M&TPs	79.6	0.27%
Veterinary Drugs, M&TPs	78.2	0.26%
Tire Additive Chemicals	41.4	0.14%
Per- and Polyfluoroalkyl Substances	6.08	0.021%

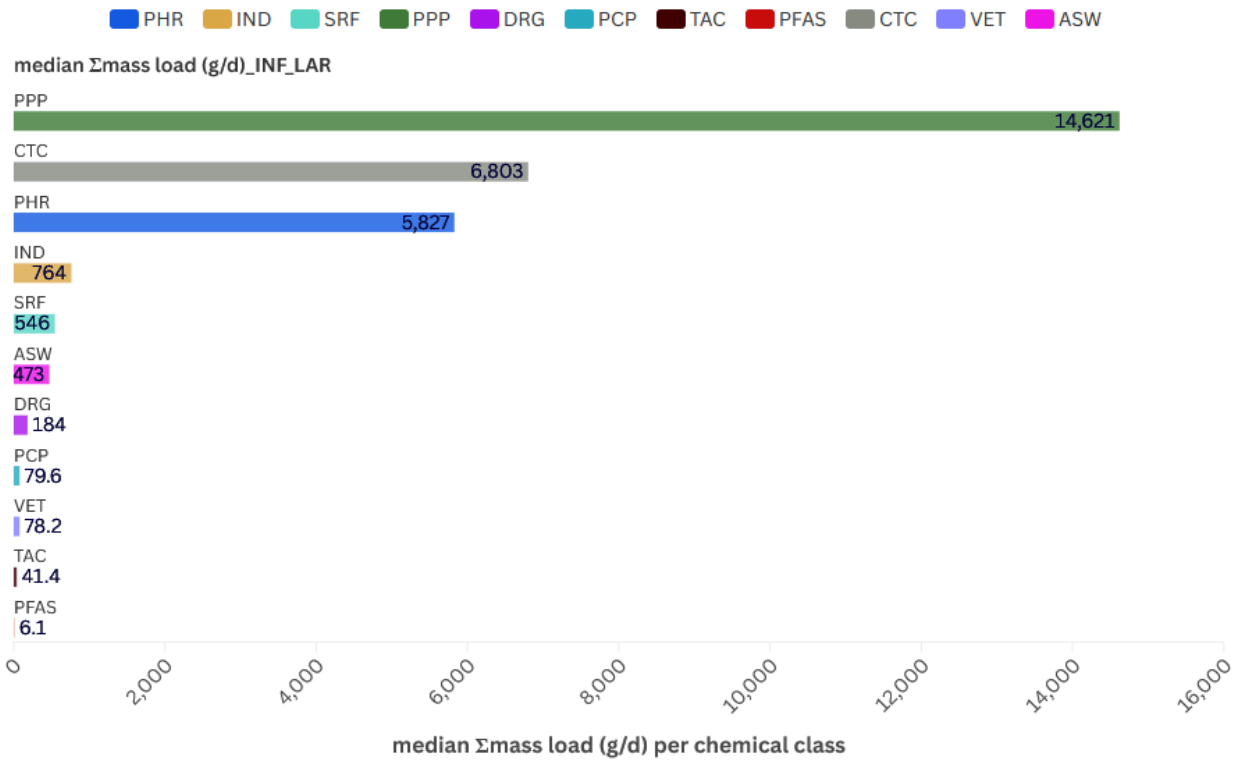

Figure 8: Median aggregated mass loads of the determined chemicals through targeted analysis in *influent* wastewater, collected from EEL WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

Influent mass load analysis from the Larissa WWTP reveals that the highest proportion of chemicals belongs to the plant protection products chemical class. This finding is in line with the main wastewater source, which enters WWTP. The Thessaly region, and in particular the area around its capital city of Larissa comprises the largest agricultural area in Greece, encompassing diverse cultivations, as well as livestock units. A high proportion of the PPP chemical load is affected by specific pesticide metabolites, like methiocarb-sulfone and methiocarb-sulfoxide, reflecting the extensive use of respective parent compounds (i.e. methiocarb; utilized in tomato and corn cultivations in the area), as well as consumption of foods relating to cultivations sprayed with specific pesticide products.

Measured concentration levels for all detected chemicals were normalized with daily WWTP flow rates and calculated as mass loads (g/day). The mass loads in **effluent** wastewater samples are depicted in the following pie chart (**Figure 9**) and in **Table 12**.

Table 12: Median aggregated mass load (g/d) and contribution of each chemical class in the total mass load in the analyzed *effluent* wastewater samples, collected from EEL WWTP.

Class	Median ∑ mass load (g/d)	Percentage
Pharmaceuticals, M&TPs	2,074	46%
Coffee and Tobacco related compounds	1,564	35%
Surfactants	235	5.3%
Plant Protection Products, M&TPs	233	5.2%
Industrial Chemicals	192	4.3%
Tire Additive Chemicals	54.7	1.2%
Personal Care Products, M&TPs	48.8	1.1%
Drugs of Abuse, M&TPs	36.3	0.81%
Veterinary Drugs, M&TPs	17.4	0.39%
Artificial Sweeteners	15.4	0.34%
Per- and Polyfluoroalkyl Substances	6.8	0.15%

Besides the total chemical load in **influent** wastewater, the **removal efficiency** of Larissa WWTP is also noteworthy. Median aggregated mass loads per chemical class in both **influent** and **effluent** wastewater samples and the **average %removal** per chemical class are summarized in **Table 13**.

As summarized in **Table 13**, pharmaceuticals exhibit notably higher removal efficiencies compared to other chemical groups, including personal care products (e.g., cosmetics, UV filters, skin cleansers and synthetic musks), and surfactants (e.g., biocides, antiseptics, detergents and cleansers). Regarding TACs, they exhibit an increase in concentration, and mass load during wastewater treatment. This chemical class presents a greater threat than other chemicals, as will be discussed below, highlighting a greater need to investigate, regarding their potential toxicity towards freshwater and marine organisms, which are greatly affected by effluent wastewater inputs from the Larissa WWTP. The chemical content of PFAS is also significantly increased (negative removal ratio equal to 18%), possibly due to larger carbon chain PFAS breakdown during the cleanup process. These smaller carbon chain PFAS should also be investigated regarding their toxicity, as they are continuously discharged into aquatic compartments.

Table 13: Median aggregated mass loads (g/d) and number of detected compounds through target analyses in the EEL WWTP case study. The average (%) removal of each chemical class is also presented on the table.

Class	Influent wastewater		Effluent wastewater		Average %removal
	Number of compounds	Median ∑ mass load (g/d)	Number of compounds	Median ∑ mass load (g/d)	
PPP	28	14,621	25	233	-98%
CTC	10	6,803	10	1,564	-77%
PHR	157	5,827	155	2,074	-64%
IND	26	764	25	192	-77%
SRF	14	546	15	235	-49%
ASW	3	473	2	15.4	-96%
DRG	18	184	14	36.3	-83%
PCP	7	79.6	6	48.8	-10%
VET	5	782	5	17.4	-80%
TAC	5	41.4	5	54.7	**+6%**
PFAS	8	6.08	9	6.8	**+18%**

The average removal values of both plant protection substances and artificial sweeteners highlight the beneficial cleanup processes regarding these chemical classes, moving them far down the list of effluent mass loads. However, their total risk value towards aquatic biota remains significantly higher than pharmaceuticals, despite their lower mass loads, as will be discussed further below.

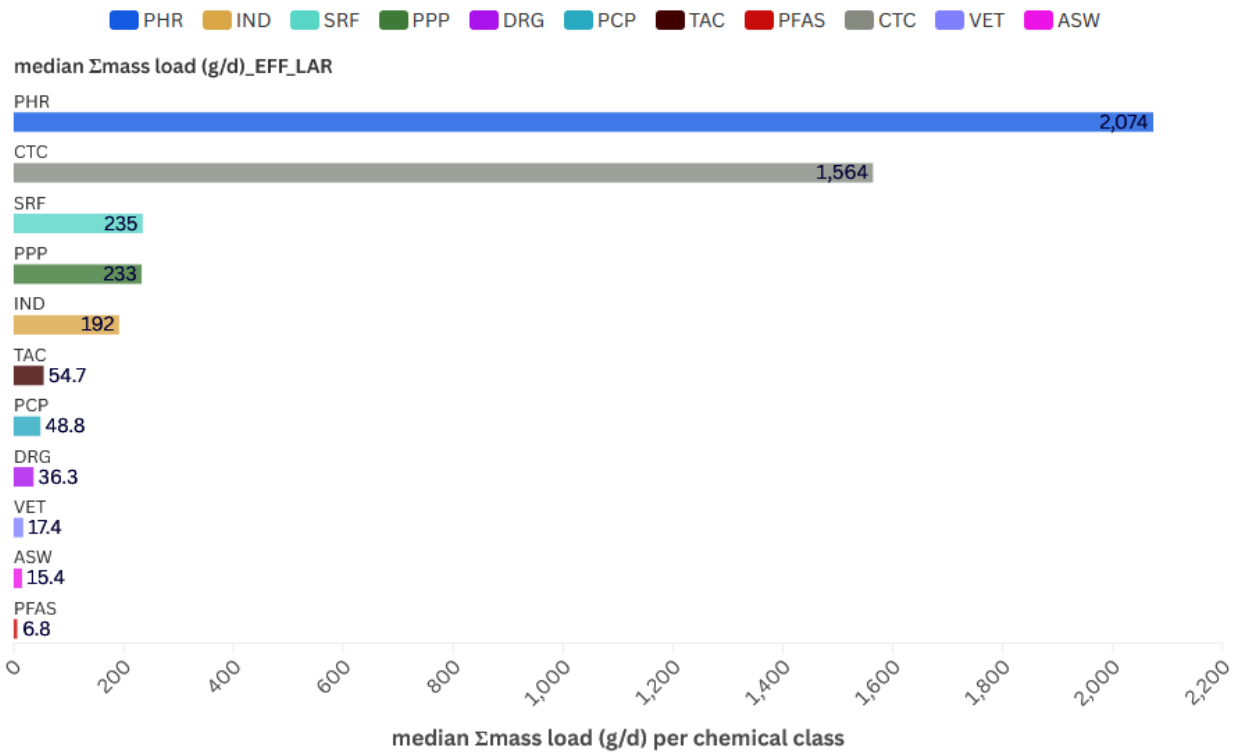

Figure 9: Median aggregated mass loads of the determined chemicals through target analysis in the *effluent* wastewater, collected from EEL WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

5.4 Results from target analysis, suspect screening & elemental analysis in Larissa WWTP

A comprehensive suspect screening workflow was carried out for the Larissa WWTP samples as before, for chemicals with no available analytical standards. The occurrence of cosmetics, surfactants, plastic related chemicals and heavy metals was investigated to amplify the covered chemical domain. Additional *28 chemicals were identified* in level 2 or 3, based on the confidence scheme proposed by Celma et al. (2020) [27]. Again, the chemical mixture presents a different content when integrating suspect screening results, especially in influent wastewater samples.

After incorporating these tentatively identified substances *via* suspect screening, the depiction of the chemical content of **influent** wastewater appears differently compared to the chemical profile for the targeted analyses, which indicated that there are many compounds that are "hidden" in the samples due to the unavailability of commercial analytical standards. In brief, a total of *339 compounds were identified in influent wastewater*. These belong to pharmaceuticals and M&TPs (46%, n=157), industrial chemicals (11%, n=37), surfactants (8.6%, n=29), plant

protection products (8.3%, n=28), (heavy) metal ions (5.9%, n=20), drugs of abuse (5.3%, n=18), TACs (4.4%, n=15), coffee and tobacco related compounds (%, n=10), personal care products, including tentatively identified cosmetics (2.9%, n=9), PFAS (2.4%, n=8), veterinary drugs (1.5%, n=5) and artificial sweeteners (0.89% each, n=3). Thus, pharmaceuticals along with their metabolites and TPs, comprise 46% of the total determined compounds.

Similarly, in **effluent** wastewater, *327 compounds were determined*, belonging to pharmaceuticals, M&TPs (47%, n=155), industrial chemicals (11%, n=35), surfactants (8.9%, n=29), plant protection products (7.6%, n=25), (heavy) metals (6.1%, n=20), TACs (4.6%, n=15), drugs of abuse (4.3%, n=14), coffee and tobacco related compounds (3.1%, n=10), PFAS (2.8%, n=9), personal care products (2.4%, n=8), veterinary drugs (1.5%, n=5) and artificial sweeteners (0.61%, n=2). Thus, the total number of pharmaceuticals does not exceed 50% of total determined compounds.

Moreover, an entirely different profile of the influent wastewater mass load is presented in **Table 14,** when suspect screening results are incorporated. As presented in **Figure 10**, *pharmaceuticals are <10% of the overall daily mass load, a similar fact as the Psyttalia WWTP*, following surfactants, plant protection products and coffee and tobacco related compounds which comprise 48%, 23% and 11% of the total chemical load, respectively.

Table 14: Median aggregated mass load (kg/d) and contribution of each chemical class in the total mass load in the analyzed *influent* wastewater samples, collected from EEL WWTP.

Class	Median aggregated mass load (kg/d)	Percentage
Surfactants	30.8	48%
Plant Protection Products, M&TPs	14.6	23%
Coffee and Tobacco related compounds	6.80	11%
Pharmaceuticals, M&TPs	5.83	9.1%
Industrial Chemicals	3.91	6.1%
Veterinary Drugs, M&TPs	0.78	1.2%
Personal Care Products, M&TPs	0.593	0.92%
Artificial Sweeteners	0.473	0.73%
Drugs of Abuse, M&TPs	0.184	0.29%
Tire Additive Chemicals	0.41	0.63%
Per- and Polyfluoroalkyl Substances	0.006	0.0093%

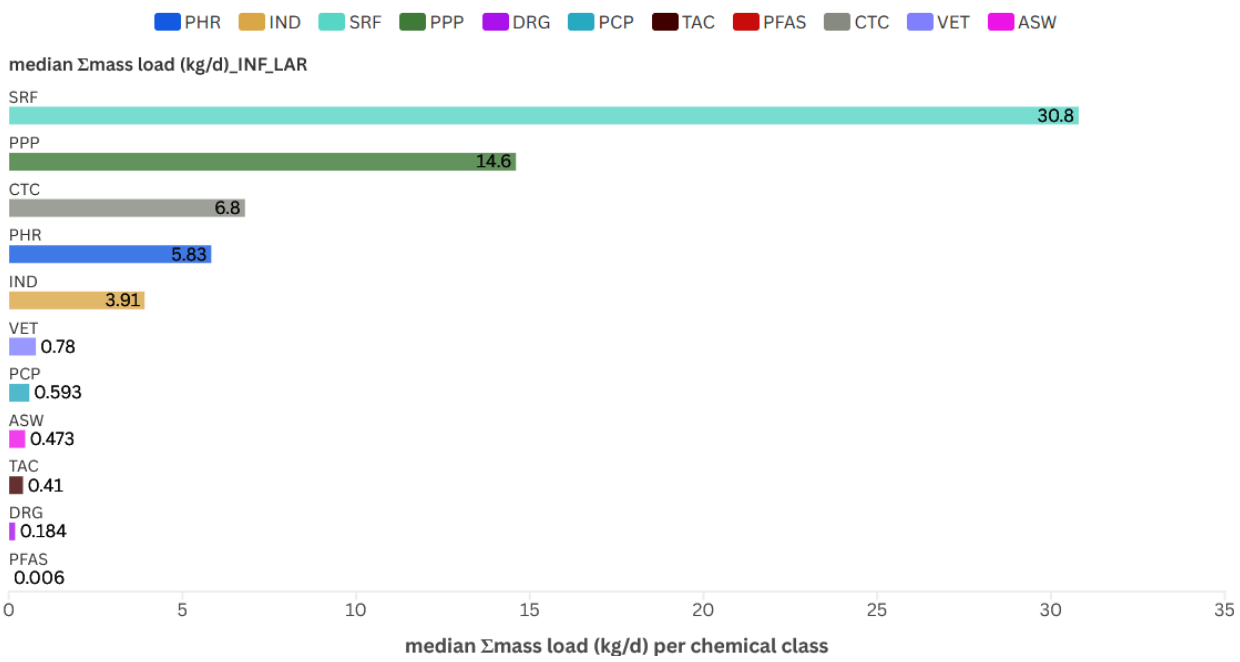

Figure 10: Median aggregated mass loads of the determined organic chemicals through target and suspect screening analysis in *influent* wastewater samples, collected from EEL WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

The median aggregated mass loads per chemical class in *effluent* wastewater, collected from EEL WWTP, are summarized in **Table 15**.

Table 15: Median aggregated mass load (kg/d) and contribution of each chemical class in the total mass load in the analyzed effluent wastewater samples, collected from EEL WWTP.

Class	Median ∑ mass load (kg/d)	Percentage
Pharmaceuticals, M&TPs	2.07	33%
Coffee and Tobacco related compounds	1.56	25%
Surfactants	1.14	18%
Industrial Chemicals	1.03	16%
Plant Protection Products, M&TPs	0.233	3.7%
Personal Care Products, M&TPs	0.134	2.1%
Tire Additive Chemicals	0.055	0.87%
Drugs of Abuse, M&TPs	0.036	0.57%
Veterinary Drugs, M&TPs	0.017	0.27%
Artificial Sweeteners	0.015	0.23%
Per- and Polyfluoroalkyl Substances	0.007	0.11%

Table 16: Median aggregated mass loads (g/d) and number of detected compounds through target suspect screening analyses in the EEL WWTP case study. The average (%) removal of each chemical class is also presented on the table. The following abbreviations were used in the table: PHR=pharmaceuticals, M&TPs, CTC=coffee and tobacco related compounds, IND=industrial chemicals, SRF=surfactants, ASW=artificial sweeteners, DRG=drugs of abuse, PPP=plant protection products, PCP=personal care products (including cosmetics), VET=veterinary drugs, TAC= tire additive chemicals, and PFAS=per-and polyfluoroalkyl substances.

Class	Influent wastewater		Effluent wastewater		Average %removal
	Number of compounds	Median ∑ mass load (kg/d)	Number of compounds	Median ∑ mass load (kg/d)	
SRF	29	30.8	29	1.14	-94%
PPP	28	14.6	25	0.233	-98%
CTC	10	6.80	10	1.56	-77%
PHR	157	5.83	155	2.07	-64%
IND	37	3.91	24	1.03	-73%
VET	5	0.78	5	0.017	-80%
PCP	9	0.593	8	0.134	-78%
ASW	3	0.473	2	0.015	-96%
DRG	18	0.184	14	0.036	-83%
TAC	15	0.41	15	0.055	**+7%**
PFAS	8	0.006	9	0.007	**+18%**

The removal efficiency is further evaluated by the integration of HRMS-based suspect screening results (**Table 16**, **Figure 11**). Target and suspect-screening data indicate that pharmaceuticals removal does not exceed 64%. Additionally, for certain chemical classes, namely PFAS and TACs, unchanged or slightly increased mass loads are found, indicating their recalcitrant nature or formation from precursors during the WWTP processes. These groups include emerging contaminants, such as 6-PPD-quinone, various benzothiazoles, and also small carbon-chain PFAS.

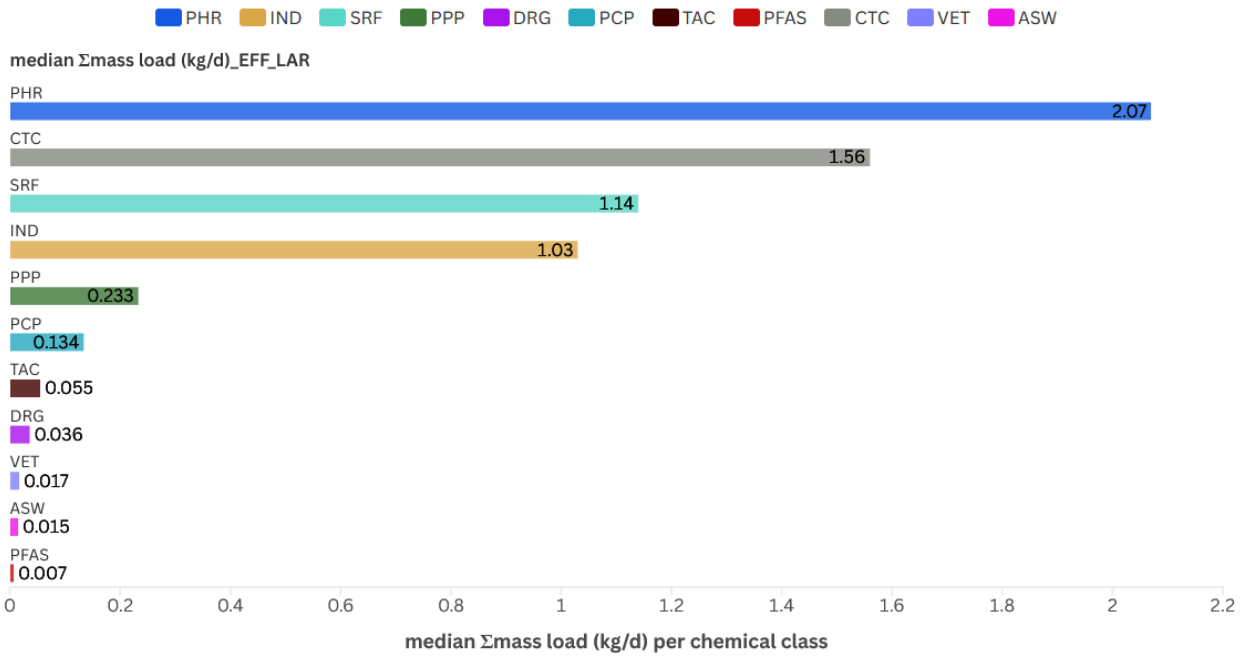

Figure 11: Median aggregated mass loads of the determined organic chemicals through target and suspect screening analysis in *effluent* wastewater samples, collected from EEL WWTP. The following abbreviations were used in the figure. IND: industrial chemicals, SRF: surfactants, PCP: personal care products, PHR: pharmaceuticals, CTC: coffee and tobacco related chemicals, ASW: artificial sweeteners, DRG: drugs of abuse, PPP: plant protection products, VET: veterinary drugs, TAC: tire additive chemicals, PFAS: per- and polyfluoroalkyl substances.

5.5 Ecotoxicological Risk Assessment

The ecotoxicological risk assessment in the effluent wastewater from Psyttalia WWTP revealed that *32 compounds, 3 out of which were identified through suspect screening*, exceeded the respective PNEC value. The compounds, exceeding the respective PNEC value, are summarized in **Table 17**. *Seventeen out of the 32 compounds had available experimental NEC values*, whereas additional 6 compounds appeared to have a high Tanimoto similarity score (>0.5). Briefly, 8 surfactants, 7 plant protection products, 5 industrial chemicals, 5 TACs, 3 pharmaceuticals, 2 veterinary drugs, 1 personal care products, and 1 artificial sweetener exceeded their PNEC values. As depicted in **Figure 12**, although the number of TAC that exceeded their PNEC values was relatively small, compared to other classes, their average weekly (%) toxic load was approximately 52%. This was mainly attributed to Hexa(methoxymethyl)melamine (HMMM), which had the highest Risk Quotient values. Plant Protection Products appeared to have an average weekly (%) toxic load equal to 26%, which was mainly attributed to Fipronil, followed by surfactants (9%), veterinary drugs (6%), industrial chemicals (4%). The contribution of the remaining chemical classes was insignificant.

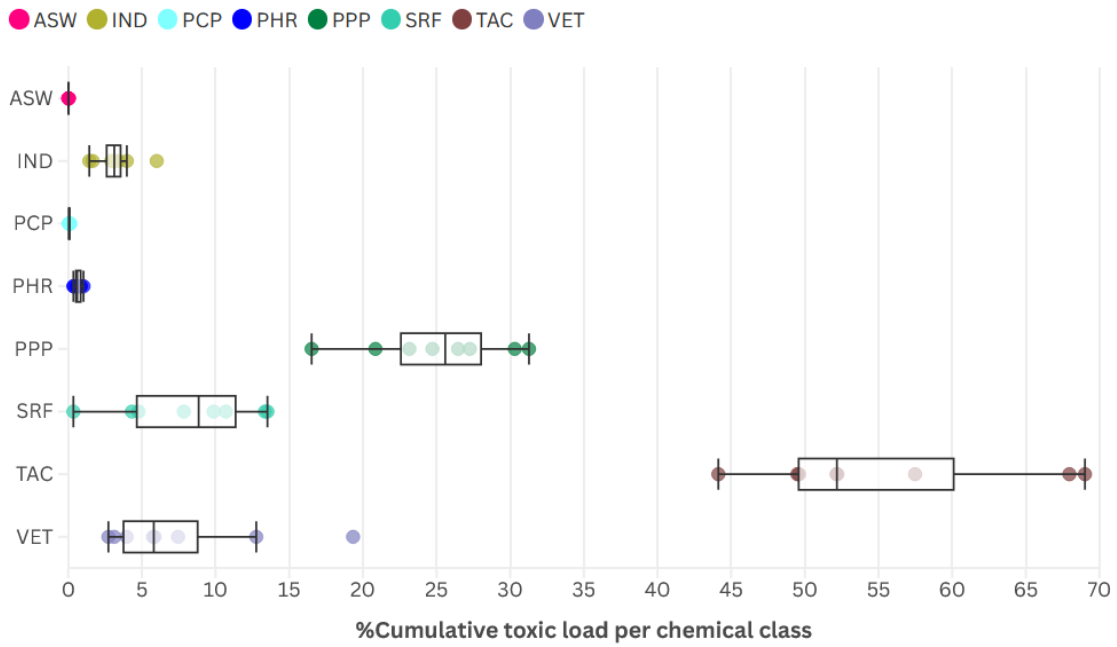

Figure 8: % Toxic load per chemical class in the Psyttalia WWTP case study.

Similarly, the ecotoxicological risk assessment in the effluent wastewater from Larissa WWTP revealed that *40 compounds, 9 out of which were identified through suspect screening*, exceeded the respective PNEC value. The compounds, exceeding the respective PNEC value, are summarized in **Table 18**. *Twenty-three out of the 40 compounds had available experimental PNEC* values, whereas additional 5 compounds appeared to have a high Tanimoto similarity score (>0.5). Briefly, 14 surfactants, 7 industrial chemicals, 5 plant protection products, 4 TACs, 4 pharmaceuticals, 3 personal care products, 2 drugs of abuse and 1 veterinary drug exceeded their PNEC values. As depicted in **Figure 13**, the average weekly (%) toxic load for plant protection products was approximately 65%, reflecting the agricultural activities of this region in Greece. This was mainly attributed to Methiocarb-Sulfone and Imidacloprid, which had the highest Risk Quotient values. TACs appeared to have an average weekly (%) toxic load equal to 22%, followed by industrial chemicals (8%), and surfactants (5%). The contribution of the remaining chemical classes was insignificant.

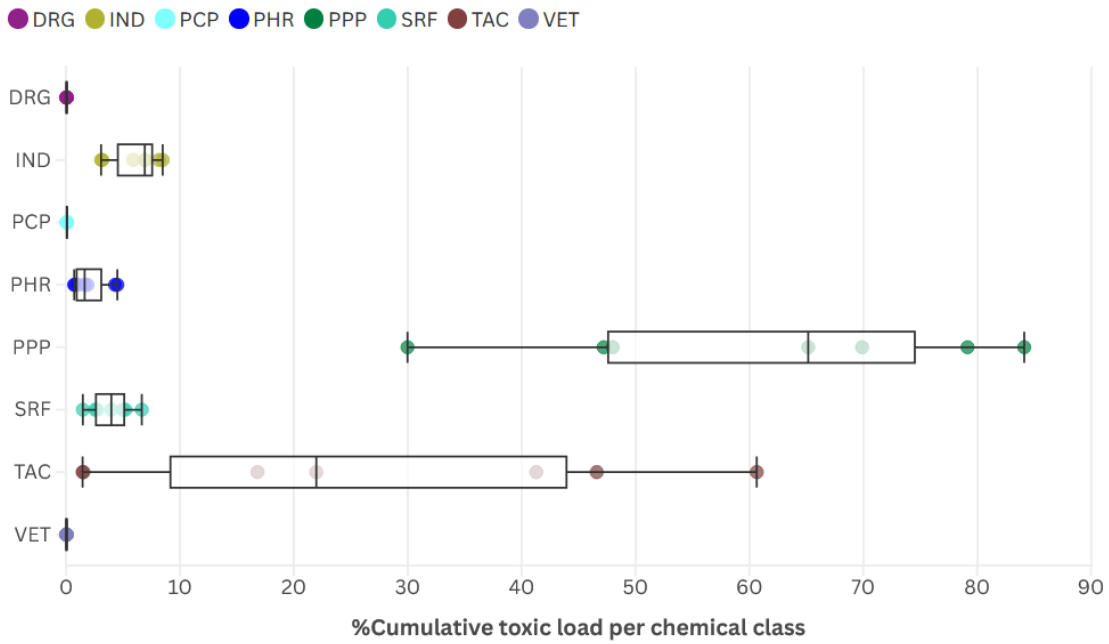

Figure 9: % Toxic load per chemical class in the Larissa WWTP case study.

Table 17: Chemicals identified in Psyttalia WWTP and exceeded the respective PNEC value. In the table, concentration and RQ range, PNEC values and the species for which the PNEC was measured, as well as the Tanimoto similarity score for chemicals without experimental NEC values, are summarized. The compounds identified through suspect screening are marked with blue color.

Analyte	*Class*	*PNEC (ng/L)*	*RQ range*	*Species at minimum*	*Tanimoto Similarity Score*
Hexa(methoxymethyl)melamine (HMMM)	***TAC***	0.219	1,225 - 6,137	*Daphnia Magna*	0.28
Fipronil	***PPP***	1.0	391 - 2,123	----	Maximum Acceptable Concentration
Fenbendazole	***VET***	7.00	102 - 485	*Ictalurus punctatus*	0.64
N,N-Dimethyltetradecylamine	***SRF***	1.80	15 - 369	*Selenastrum capricornutum*	experimental
N,N-Dimethyldodecylamine N-oxide	***SRF***	110	5.7 - 205	*Selenastrum capricornutum*	experimental
Methyl 3-bicyclo[2.2.1]hept-5-en-2-yl-3-methyloxirane-2-carboxylate	***IND***	420	120 - 151	*Oncorhynchus mykiss*	0.23
N,N-Dimethyltetradecylamine-N-oxide	***SRF***	110	3.4 - 106	*Selenastrum capricornutum*	experimental
N-(1.3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-quinone)	***TAC***	0.640	33 - 49	*Oncorhynchus mykiss*	experimental
Pirimiphos-methyl	***PPP***	0.50	5.6 - 42	*not reported*	experimental
Rifaximin	***PHR***	21.3	8.9 - 29	*Lepomis macrochirus*	0.17
Albendazole oxide	***VET***	5.00	2.1 - 27	*Ictalurus punctatus*	0.54
N,N-Dimethyldodecylamine	***SRF***	82.9	0.58 - 17	*Daphnia Magna*	experimental
Azoxystrobin	***PPP***	200	0.0 - 15	----	Maximum Acceptable Concentration
N-Desmethylvenlafaxine (Norvenlafaxine)	***PHR***	7.50	7.8 - 9.0	*Pimephales promelas*	0.33
Benzododecinium	***SRF***	110	0.31 - 6.7	*Daphnia Magna*	experimental
N,N'-Diphenyl-1,4-phenylenediamine (DPPD)	***TAC***	20.0	4.1 - 5.8	*Leuciscus idus ssp. melanotus*	0.53
Clarithromycin	***PHR***	130	2.1 - 5.3	----	Annual Average Quality Standard
Hexadecyltrimethylammonium	***SRF***	4.99	2.1 - 4.7	*Selenastrum capricornutum*	experimental
Methyl 4(or 1)-isopropyl-1(or 4)-methylbicyclo[2.2.2]oct-5-ene-2-carboxylate	***IND***	420	1.0 - 4.5	*Oncorhynchus mykiss*	0.24
di-t-Butylhydroquinone	***IND***	1899	0.55 - 4.3	*Selenastrum capricornutum*	0.59
Minoxidil	***PCP***	37.0	1.8 - 4.2	*Gambusia affinis*	0.17
Fluometuron	***PPP***	320	0.1 - 3.3	*not reported*	experimental
4-Nitrophenol	***IND***	92.0	3	*Cyprinus carpio*	experimental
2-Hydroxybenzothiazole (2-OHBT)	***TAC***	167	1.5 - 2.9	*Oncorhynchus mykiss*	0.36
Chlorantraniliprole	***PPP***	250	0.2 - 2.0	*Chironomus riparius*	experimental
Benzyldimethyltetradecylammonium	***SRF***	110	0.35 - 1.9	*Daphnia Magna*	experimental
2,2,4-Trimethyl-1,2-dihydroquinoline (RD)	***TAC***	699	1.3 - 1.7	*Selenastrum capricornutum*	0.50

Analyte	*Class*	*PNEC (ng/L)*	*RQ range*	*Species at minimum*	*Tanimoto Similarity Score*
Difenoconazole	***PPP***	360	0.2 - 1.5	----	Maximum Acceptable Concentration
Sucralose	***ASW***	2712	0.74 - 1.3	*Selenastrum capricornutum*	0.26
Azoxystrobin acid	***PPP***	197	0.45 - 1.2	*Daphnia Magna*	0.83
Trimethyloctylammonium	***SRF***	22.0	0.68 - 1.1	*Daphnia Magna*	experimental
trans-8-(1-Methylethyl)-1-Oxaspiro[4.5]decan-2-one	***IND***	18000	0.66 - 1.1	*Pimephales promelas*	0.23

Table 18: Chemicals identified in Larissa WWTP and exceeded the respective PNEC value. In the table, concentration and RQ range, PNEC values and the species for which the PNEC was measured, as well as the Tanimoto similarity score for chemicals without experimental PNEC values, are summarized. The compounds identified through suspect screening are marked with blue colour.

Analyte	*Chemical class*	*PNEC (ng/L)*	*RQ range*	*Species at minimum*	*Tanimoto Similarity Score*
Methiocarb-sulfone	***PPP***	1.80	1,170 - 2,660	*Daphnia Magna*	0.60
Hexa(methoxymethyl)melamine (HMMM)	***TAC***	0.219	13 - 2,540	*Daphnia Magna*	0.28
Diphosphocine	***IND***	15.0	133 - 283	*Oryzias latipes*	0.15
Imidacloprid	***PPP***	7.00	122 - 276	----	Annual Average Quality Standard
N-(1.3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-quinone)	***TAC***	0.640	8.6 - 156	*Oncorhynchus mykiss*	experimental
Hexadecyltrimethylammonium	***SRF***	4.99	0.28 - 138	*Selenastrum capricornutum*	experimental
Rifaximin	***PHR***	21.3	15 - 75	*Lepomis macrochirus*	0.17
N,N-Dimethyldodecylamine-N-oxide	***SRF***	110	12 - 70	*Selenastrum capricornutum*	experimental
C13-LAS	***SRF***	936	5.9 - 52	*Daphnia Magna*	experimental
N,N-Dimethyltetradecylamine-N-oxide	***SRF***	110	6.0 - 29	*Selenastrum capricornutum*	experimental
1.2.3.6-Tetrahydrophthalimide (cis-)	***PPP***	37.0	6.86 - 27	*Carassius auratus*	0.35
Clindamycin	***PHR***	44.0	5.2 - 23	----	Maximum Acceptable Concentration
4-Nitrophenol	***IND***	92.0	1.3 - 13	*Cyprinus carpio*	experimental
N,N-Dimethyldodecylamine	***SRF***	82.9	0.98 - 13	*Daphnia Magna*	experimental
N,N-Dimethyldecylamine	***SRF***	1.80	0.82 - 12	*Selenastrum capricornutum*	experimental
Diisobutyl phthalate	***IND***	1799	4.1 - 9.6	*Selenastrum capricornutum*	experimental
C13-AS	***SRF***	10236	0.041 - 7.7	*Selenastrum capricornutum*	experimental
C14-SAS	***SRF***	414	0.0 - 6.8	*Daphnia Magna*	experimental
Trimethyloctylammonium	***SRF***	22.0	0.93 - 6.2	*Daphnia Magna*	experimental
Benzyldimethylhexadecylammonium	***SRF***	110	0.073 - 5.9	*Daphnia Magna*	0.41
Cetyl phosphate	***IND***	1400	2.6 - 5.7	*Oryzias latipes*	0.63
DEHP [Bis-(2-ethylhexyl) phthalate]	***IND***	1300	0.8 - 5.6	----	Annual Average Quality Standard
Fipronil-sulfone	***PPP***	63.1	2.8 - 5.3	*Oryzias latipes*	experimental
Clarithromycin	***PHR***	130	1.4 - 5.0	----	Annual Average Quality Standard
C15-SAS	***SRF***	397	0.20 - 5.0	*Daphnia Magna*	experimental
C11-SAS	***SRF***	495	0.0 - 4.1	*Daphnia Magna*	experimental
Benzododecinium	***SRF***	110	0.31 - 3.7	*Daphnia Magna*	experimental

Analyte	*Chemical class*	*PNEC (ng/L)*	*RQ range*	*Species at minimum*	*Tanimoto Similarity Score*
Triphenyl phosphate	***IND***	170	0.20 - 3.5	*not reported*	experimental
Benzyldimethyltetradecylammonium	***SRF***	110	0.14 - 3.3	*Daphnia Magna*	experimental
Fipronil-sulfide	***PPP***	57.5	1.5 - 2.6	*Oryzias latipes*	experimental
Ipratropium	***PHR***	3.30	0.49 - 2.6	*Lepomis macrochirus*	0.28
Triclosan	***PCP***	20	0.5 - 2.3	----	Annual Average Quality Standard
Fenbendazole	***VET***	7.00	0.15 - 2.3	*Ictalurus punctatus*	0.64
Benzophenone-7	***PCP***	1392	0.43 - 1.7	*Danio rerio*	0.53
Norcocaine	***DRG***	440	0.27 - 1.5	*Selenastrum capricornutum*	0.33
2-Hydroxybenzothiazole (2-OHBT)	***TAC***	167	0.25 - 1.5	*Oncorhynchus mykiss*	0.36
11-nor-9-carboxy-THC (THC-COOH)	***DRG***	26.0	0.15 - 1.2	*Daphnia Magna*	0.29
2-Hexyl-1,3-dioxolane-4-methanol	***PCP***	1500	0.0 - 1.1	*Oncorhynchus mykiss*	0.36
N,N'-Diphenyl-1,4-phenylenediamine (DPPD)	***TAC***	20.0	0.11 - 1.1	*Leuciscus idus ssp. melanotus*	0.53
Benzothiazole-2-sulfonic acid	***IND***	420	0.47 - 1.0	*Oncorhynchus mykiss*	0.47

6. Conclusions and recommendations

Urban wastewater represents a major conduit for the release of diverse chemical classes into aquatic environments across Europe. The updated Urban Wastewater Treatment Directive (2024/3019/EU) introduces a more comprehensive and forward-looking regulatory framework, mandating the monitoring of micropollutants in influent, effluent, and receiving waters. Within this context, the present study provides an in-depth characterization of the chemical fingerprint of influent and effluent wastewater from the Psyttalia (Athens) and Larissa WWTPs, employing highly sensitive and validated analytical methodologies, advanced instrumentation, and modern chemoinformatic tools.

The applied analytical methodologies enabled the detection of thousands of substances, spanning a broad spectrum of chemicals of priority concern identified through both policy frameworks and emerging research. The analysis of influent wastewater offers valuable insights into societal consumption and disposal patterns, while the evaluation of the Psyttalia (Athens) - one of the largest facilities in Europe and worldwide - and Larisa WWTPs provides a robust assessment of removal efficiencies for the determined pollutants. Furthermore, the ecotoxicological risk assessment using experimental PNEC values identifies compounds discharged into the Saronic Gulf and Pineios River with the potential to pose ecological risks to aquatic organisms and the wider aquatic environment.

The results demonstrate that wastewater from two representative WWTPs in Greece contain a remarkably diverse chemical mixture. Although more than 100 pharmaceuticals and Metabolites & Transformation Products (M&TPs) were detected, *pharmaceuticals accounted for less than 40% of the total mass load in influent wastewater in both case studies*. The consistent ratios between pharmaceuticals and their metabolites further confirm that their presence is primarily due to human consumption rather than direct disposal. Suspect-screening analyses also revealed the presence of numerous compounds, lacking available analytical standards, underscoring the complexity and underexplored nature of wastewater chemical mixtures. Notably, *pharmaceuticals contributed only about 2% of the overall chemical burden* (based on median aggregated influent mass loads), whereas cosmetics, surfactants, and industrial chemicals exhibited substantially higher total mass loads. Moreover, *the toxic loads of the determined pharmaceuticals in effluent wastewater samples were relatively low, compared to other chemical classes, such as tire additive chemicals and plant protection products*.

These findings collectively suggest that current European legislation - focused mainly on pharmaceuticals and benzotriazoles - captures only a fraction of the chemical groups contributing to the total pollutant (and toxic) load in urban wastewater. Consequently, regulatory bodies within the European Union should consider revising and expanding existing directives to encompass a broader range of chemical classes. The widely cited assumption that pharmaceuticals constitute more than half of the chemical burden entering WWTPs is not supported by empirical, reliable

experimental mass-load data and is effectively challenged by modern (HR)MS-based analyses and advanced chemometric and semi-quantification workflows.

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