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Advanced on-site monitoring of industrial wastewater: integration of online biological and chemical tools to identify toxic compounds

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ABSTRACT

Monitoring of wastewater treatment plant (WWTP) effluents from complex industrial clusters with high temporal resolution is crucial for detecting and subsequently managing problematic compounds to reduce their release into the environment. This study explored the potential of combining biological early warning systems (BEWS) with a transportable high-performance liquid chromatography coupled with electrospray ionization and high-resolution tandem mass spectrometry (HPLC-ESI-HRMS/MS) platform (MS2Field) to detect and identify toxic pollutants in industrial-driven WWTP effluent. BEWS, using the organisms *Daphnia magna, Chlorella vulgaris*, and *Gammarus pulex*, provided real-time biological responses to micropollutants, while the MS2Field allowed continuous chemical detection of toxic compounds in parallel. Over a two-month monitoring period, significant correlations were observed between behavioural changes in the BEWS organisms and the presence of industrial target and non-target substances in the WWTP effluent. The parallel measurement and correlation of biological and chemical time series revealed four toxicity events and identified eight known and unknown compounds or compound classes associated with these toxicity peaks. Together with information from the industrial production site, this integrated approach enabled strategic source tracing of industrial emissions. When comparing data from the online monitoring tools with results from laboratory bioassays and chemical analysis of composite samples, it became obvious that high temporal resolution measurements are the key to accurately indicate toxicity trends. Otherwise, contaminant peaks were partially masked by dilution or degradation during storage. The approach offers traceability of sources for industries and regulators seeking to implement more effective and sustainable pollution management strategies.

1. Introduction

Industrial discharges are highly dynamic and contain a mixture of known and unknown compounds, some of which exhibit problematic potential for the aquatic environment. These compounds originate from various production processes and often persist during treatment (Rogowska et al., 2020). In Switzerland, monitoring of industrial wastewater treatment plants (WWTPs) is regulated by the Water Protection Ordinance (WPO), focusing on sum parameters like total organic carbon (TOC) and chemical oxygen demand (COD), along with specific target compounds set by cantonal standards (Schärer et al., 2014). The overall policy on toxic substances is grounded in principles such as self-regulation, the use of best available techniques, and source-specific measures (Wunderlin and Gulde, 2022). Sampling strategies for industrial wastewater typically involve grab or composite sampling. These methods require significant resources, especially in areas with diverse industries and constantly changing production processes (Anliker et al., 2020). Additionally, composite sampling may mask short-term emission peaks and may lead to compound degradation during storage, complicating accurate source identification (Aymerich et al., 2017). Analysis of wastewater samples is commonly performed using chemical methods such as liquid chromatography-mass spectrometry (LC-MS) and gas

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chromatography-mass spectrometry (GC–MS) to identify known target compounds (Kadadou et al., 2024; Loos et al., 2024). In some cases, laboratory bioassays are employed alongside chemical measurements, to assess effects of WWTP effluents on aquatic organisms (Kienle et al., 2019; Neale et al., 2017; Cavallin et al., 2021) or used in routine monitoring of industrial effluents (OSPAR Commission, 2000). While these laboratory-based methods provide valuable insights, they are often time-consuming and may not effectively capture the rapid fluctuations in contaminant concentrations found in industrial wastewater effluents (Sabotič et al., 2024).

To precisely detect and identify industrial pollutants in dynamic wastewater effluent, a high-resolution and continuous monitoring approach would be needed as suggested by Anliker et al. (2022) and demonstrated for urban wastewater by Kizgin et al. (2024). Online monitoring tools, such as biological early warning systems (BEWS) or high-performance liquid chromatography coupled with electrospray ionization and high-resolution tandem mass spectrometry (HPLC-E-SI-HRMS/MS), or ideally a combination of both, could complement traditional methods of industrial wastewater monitoring, particularly in cases where dynamic known and unknown toxic pollutants occur and source identification is warranted (Bownik and Wlodkowic, 2021; Stravs et al., 2021). These tools address the shortcomings of grab and composite sampling followed by off-line chemical analysis and bioassays by enabling continuous, real-time observation and identification of problematic emissions on-site. The online analysis can be complemented by off-line non-target screening (NTS) of the collected data (Hollender et al., 2017). For NTS, chemical signals in a sample are elucidates using compound databases and spectra libraries that suggest candidate structures for unknown pollutants (Bletsou et al., 2015). NTS has proven to be particularly valuable in wastewater monitoring, where transformation products of pesticides and pharmaceuticals are often found (Wang et al., 2020). In combination with BEWS, NTS can strengthen the ability to uncover the pollutants that are driving the observed toxicological effects in the BEWS.

Previous studies (De Hoogh et al., 2006; Hug et al., 2015; Anliker et al., 2020; Kizgin et al. 2024) highlight the importance of time-series analysis and aligning toxicity measurements with chemical analysis to successfully identify short-term peaks of harmful contaminants. Building on this foundation, the present study applies and further develops the approach in the context of industrial wastewater which is a matrix characterized by pronounced temporal variability, a complex chemical composition, and site-specific discharge patterns. The aim was to assess the added value of real-time monitoring for detecting short-term toxicity events in wastewater effluent and for prioritizing potential toxicants. Rather than focusing on the identification of individual substances, which are often proprietary and subject to confidentiality, this study emphasizes the applicability and practical relevance of the integrated monitoring strategy to support future source control efforts.

To compare the online measurements from the BEWS and the MS2Field system, a transportable HPLC-ESI-HRMS/MS platform (Stravs et al., 2021), weekly composite samples were collected and analysed using conventional monitoring methods over the same seven-week period. These included standardised laboratory bioassays with microalgae and aquatic macrophytes, as well as targeted chemical analysis. The specific objectives of this study were: (i) To implement and evaluate the integration of BEWS and online HRMS monitoring under routine industrial conditions, using continuous sampling and high-frequency data acquisition in a dynamic effluent setting. (ii) To evaluate the added value of the integrated BEWS–HRMS approach by comparing it with conventional monitoring methods, including standardized laboratory bioassays and targeted chemical analysis, for industrial wastewater surveillance. (iii) To refine the chemical–biological linkage through time-series changepoint detection and complementary correlation analyses (Pearson and Spearman), in order to improve the prioritisation of candidate toxicity drivers in complex effluents.

2. Materials and methods

2.1. Information on WWTP

This study was conducted from August 11 to September 27, 2022, at a full-scale WWTP located in Switzerland, designed to treat wastewater from up to 380′000 population equivalents. The WWTP primarily processes industrial wastewater, which constitutes approximately 2/3 of the total influent flow (16′000 m^3/day), with the remaining 1/3 derived from municipal sources. The industrial wastewater predominantly originates from chemical and pharmaceutical production processes of an industrial cluster. The treatment facility is equipped with a pretreatment system to remove solids before further processing. Following pretreatment, the wastewater undergoes primary treatment to settle and remove suspended solids. The secondary treatment stage employs an activated sludge process, which facilitates the biological degradation of organic matter as well as nitrification. Additionally, the WWTP includes phosphate removal as a tertiary treatment stage before discharge. Due to non-disclosure agreements, the locations of the WWTP and the name of the associated industrial cluster have been anonymised. Instead of specific compound names, only the substance classes reportedly processed by this industrial cluster are indicated.

Water quality indicators, including conductivity, temperature, pH, and oxygen levels of the treated wastewater, were monitored continuously at 5-minute intervals using a Multi 3320 device (WTW, Germany). Daily measurements for nitrite, nitrate, and ammonium concentrations were provided by the WWTP operators. Details on the monitoring parameters are available in Table B1 and B2 in the Supplementary Material B (SI-B).

2.2. Chemical analysis

2.2.1. Online analysis with the MS2field

The screening of industrial wastewater effluent targeted 32 specific industrial compounds alongside a collection of standard wastewater markers, which included pharmaceuticals, pesticides, industrial chemicals and their transformation products. These compounds were selected based on their relevance to municipal and industrial wastewater, suitability for high-performance liquid chromatography coupled with electrospray ionization and high-resolution tandem mass spectrometry (HPLC-ESI-HRMS/MS) analysis with the MS2Field (Stravs et al., 2021), and the availability of reference standards (STDs). All STDs and isotopically labelled internal standards (ILIS) were prepared as specific mixtures and stored at −20 °C until use. To improve recovery and ensure reproducibility, the mixtures were prepared using Evian mineral water. Evian water was used due to its consistent mineral content and low organic background, which improves reproducibility and analyte recovery by minimizing matrix effects (Schorr et al. 2024). The ultrapure water utilized in the study was obtained from an Arium Pro ultrapure water system (Sartorius, Germany), and the methanol used was of LC/MS grade (Optima, Fisher Chemical, USA). A comprehensive list of the selected ILIS is provided in Table B1–3 in the Supporting Information B (SI-B).

For HPLC-ESI-HRMS/MS analysis, wastewater effluent was directed to the MS2Field trailer, where a filtered sample (250 μL; mesh size 3 - 5 μm) was collected every 20 min. For routine analysis, each wastewater sample was mixed with ILIS solution and a nanopure:Evian water mixture (80:20) using a dilutor syringe. Separate spiked samples containing reference standards (STD) were prepared to assess recovery and compound identification. To remove particulate matter, the mixture was passed through a self-packed pre-column (stainless steel, 2.1 × 20 mm, BGB Analytik AG, Switzerland) filled with Atlantis T3 material (10 μm, Waters, Ireland). Analytes were then separated on a reverse-phase analytical column (Atlantis T3, 5 μm, 3.0 × 50 mm, Waters, Ireland) and eluted (300 μL/min) using a gradient of nanopure water and methanol, both acidified with 0.1 % formic acid. Data acquisition was conducted on a hybrid quadrupole-Orbitrap mass spectrometer (Q ExactiveTM HF with ESI source, Thermo Fisher Scientific, U.S.). The HPLC-ESI-HRMS/MS instrument operated in MS1 full-scan mode (resolution: 120′000 at *m/z* 200) with data-dependent MS2 (resolution: 30′000 at *m/z* 200) and polarity switching (−3.0, 4.0 kV). To ensure accuracy, a calibration series of 10, 25, 50, 100, 250, 500, 1000, 2500, 5000 and 10′000 ng/L was prepared and injected into the system weekly using an nanopure:Evian water mixture (80:20). Over the 7-week monitoring period, 2951 samples were processed and analysed. Candidate compounds were filtered based on mass accuracy (<5 ppm), isotopic pattern matching, and spectral similarity scores (>70 %). Blank samples and replicates were used to exclude artefacts and noise. Identifications were assigned confidence levels following the framework by Schymanski et al. (2014), ranging from Level 1 (confirmed with reference standards) to Level 3 (tentative identification based on exact mass, isotopic pattern, and MS/MS fragmentation). Details on the MS2Field are available in section 6 in the SI-A.

2.2.2. Laboratory analysis of composite samples

Weekly composite samples from the WWTP were collected for both chemical analysis and bioassay testing. WWTP staff used an autosampler to collect time-proportional samples. Daily, 50 mL aliquots were split into two 100 mL glass bottles, stored at 4 °C and −20 °C, respectively. At the end of each week, the samples stored at 4 °C were used for immediate chemical analysis and laboratory bioassays. For chemical analysis, composite samples were sent to an external ISO/IEC 17,025 certified laboratory. The laboratory employed advanced methods, including LC-MS for target analysis of known compounds. However, due to confidentiality agreements, specific analytical details cannot be disclosed.

Meanwhile, the samples stored at −20 °C were packed in ice and transported to the Eawag laboratory, where they were kept in the dark at −20 °C until analysis, which was performed six months after the sampling campaign concluded. For the analysis at Eawag, the same analytical column as in the MS2Field was used and samples were processed using an Orbitrap instrument (Q Exactive™ Plus, Thermo Fisher Scientific). Quantification of individual compounds was conducted using standard calibration curves using isotopically labeled internal standards (ILIS) and a linear regression model in TraceFinder 5.1 (Thermo Fisher Scientific Inc., 2023). For calibration, a series of standards prepared in nanopure:Evian water mixture (80:20) at concentrations of 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, and 10′000 ng/L was analyzed with the LC—HRMS/MS system. Quantification relied on the area ratio from extracted ion chromatograms (EIC) of their de-protonated or protonated molecular ions (± 5 ppm) between each analyte and its structurally identical isotope-labeled internal standard. If no identical ILIS was available, an internal standard with similar retention time and ionization characteristics was used. Target compounds were confirmed by comparing retention time (RT) of the EIC, isotopic patterns (MS1) and MS/MS spectra against reference standards. Details of the analytical concentrations are available in section 7 in the SI-A.

2.3. Bioanalysis

2.3.1. Online analysis with biological early warning systems (BEWS)

The BEWS measurements were performed as published in Kizgin et al. (2024). A battery of BEWS was employed to continuously monitor wastewater effluent: the Algae Toximeter (BBE Moldaenke) with *C. vulgaris*, to detect photosynthesis inhibition via fluorescence measurements, 2) the DaphTox II (BBE Moldaenke) with *D. magna* to detect effects on the organism's behaviour via video tracking and 3) the Sensaguard (REMONDIS Aqua) with *G. pulex* to detect effects on behaviour via impedance sensors. A detailed description of the toxicity parameters for the three BEWS is provided in Kizgin et al. (2023). The WWTP effluent was diluted with groundwater in a 50:50 ratio for monitoring with *D. magna* and *G. pulex* due to partially elevated ammonia levels and used undiluted for the Algae Toximeter, as ammonia levels won't impact the algae during the short measurement time. To reduce the need for frequent cleaning, a 0.1 µm ultrafiltration system (PC 7, PVDF, pore size: 0.08 µm, BlueFootMembranes, Belgium) was installed before the BEWS (Kizgin et al., 2023), connecting it directly to the secondary clarifier of the WWTP for continuous wastewater supply. Weekly maintenance, including organism replacement, was performed. More details on organisms, BEWS, and the filtration system are provided in Sections 1–3 in the SI-A.

2.3.2. Laboratory bioassays on composite samples

Ecotoxicological tests on the weekly composite samples of the effluent were conducted by the laboratory Soluval Santiago (Couvet, Switzerland) on behalf of the industrial cluster connected to the WWTP. Chronic toxicity tests were conducted on green algae (*Raphidocelis subcapitata*, growth, 72 h, ISO 8692, International Organization for Standardization, 2012) and macrophytes (*Lemna minor*, population growth, 7 d, ISO 20079, International Organization for Standardization, 2005). While a direct comparison to the BEWS organisms would have been valuable, such as including a standardized *Daphnia magna* acute toxicity test, this was beyond the scope of the routine testing framework applied in this study.

2.4. Interpretation of biological and chemical online analysis

To effectively compare BEWS toxicity profiles with MS2Field chemical profiles, it is crucial to establish clear criteria for evaluating data to ensure accurate interpretation of biological responses. Industrial effluents are highly complex and can interfere with both chemical and biological measurements, leading to false positives or negatives. To mitigate these challenges, a structured decision-making framework, previously developed in our previous work (Kizgin et al., 2024), was applied in this study. The framework systematically addresses potential confounding factors by first excluding technical issues such as water supply interruptions. Key physicochemical parameters, including pH, conductivity, temperature, dissolved oxygen, and ammonia levels, were then continuously monitored to discern whether deviations in organism behaviour were caused by fluctuations in water quality rather than chemical toxicity. For chemical analysis, matrix effects such as signal suppression or enhancement in high-resolution mass spectrometry were minimized by using ILIS, ensuring more reliable quantification despite the complex effluent matrix. To further elucidate the causes of the observed biological responses, time-resolved chemical profiles from target analysis and non-target screening (NTS) of significant MS signals were evaluated. (more details on the NTS process are provided in section 6.6 in SI-A). The BEWS delayed response to chemical signals, attributed to the ultrafiltration residence time of wastewater (2–3 hours), was taken into account during data interpretation.

2.5. Time series correlation of biological and chemical results

All data were analysed and visualized using the statistical software R, version 4.5.0 for Windows (R Core Team, 2025). In the case of DaphTox II and Algae Toximeter, the statistical data analysis has already been integrated into the algorithms of the systems. Therefore, no additional statistical tests were performed on these behavioural data.

To assess temporal associations between BEWS responses and chemical profiles, both Pearson and Spearman correlation coefficients were calculated. While Pearson was used to evaluate linear relationships, Spearman rank correlation was included to account for potential non-linear, monotonic associations and to provide robustness against outliers and non-normal data distributions. The analysis was performed using the cor.test function in R version 4.5.0 (R Core Team, 2025) with the Pearson method, which was chosen due to its suitability for measuring linear associations in continuous data. Correlation strengths were interpreted using standard thresholds commonly applied to both Pearson and Spearman coefficients, where values ≥0.7 were considered strong, 0.5–0.7 moderate, 0.3–0.5 weak, and <0.3 negligible (Mukaka, 2012).

Additionally, statistical significance of the correlations was tested using a two-tailed test, and p-values were extracted to assess the likelihood that the observed relationships occurred by chance.

The Sensaguard system applies an algorithm that compares short-term and long-term moving averages of behavioural activity, generating the sum parameter AlarmSum. To identify significant shifts in *G. pulex* behavioural activity, changepoint detection was applied to the 30-point moving average of the AlarmSum time series, reducing short-term noise. The Pruned Exact Linear Time (PELT) algorithm (Killick et al., 2012) was used to detect changes in mean levels. To avoid spurious detections, results were filtered using a penalty value of 1000, a minimum segment length of 50, and a mean difference threshold of >15 units. This approach allowed for robust detection of substantial behavioural shifts, guiding subsequent targeted and non-targeted chemical screening. More details about statistics are provided in section 5 in SI-A.

3. Results and discussion

3.1. Toxicity parameters of BEWS at the industrial WWTP

The toxicity parameters of *C. vulgaris* (BBE Algae Toximeter), *D. magna* (BBE DaphTox II), and *G. pulex* (REMONDIS Sensaguard) during the operation at the industrial WWTP from August 11 to September 27, 2022 are summarized in Fig. 1A-C. For *C. vulgaris*, a significant inhibition of photosynthesis was observed between September 12 and 14, where the threshold for this parameter was exceeded during effluent monitoring (Fig. 1A). A power supply disruption occurred on September 11, which may have masked the exact time point of the increase in photosynthesis inhibition. As a result, the precise start of this inhibition could not be definitively determined. For *D. magna*, the Toxic Index threshold was exceeded on two occasions: from September 9 to 13, and from September 25 to 27 (Fig. 1B). In the case of *G. pulex*, a typical diurnal rhythm in behaviour was consistently observed throughout the monitoring period (Fig. 1C). This is consistent with previous observations (Kizgin et al., 2023). These regular day-night fluctuations in activity pose a challenge for alarm generation, as applying a fixed static threshold would result in frequent false positives, particularly during natural nocturnal activity peaks that are not toxicologically relevant. Rather than relying on a fixed alarm threshold, we focused on identifying the time point when the behaviour deviated most significantly from its established trend. During the first four weeks, the alarm parameter showed a steady and gradual increase, respectively, culminating just before the weekly organism exchange. Between September 12 and 13 the alarm parameter remained elevated, with a noticeable behavioural shift indicated by the vertical red line in Fig. 1C. To support this interpretation, we applied a statistical change point detection method to the smoothed AlarmSum data, which confirmed the significance of the behavioural shift detected in Week 5. The raw data further corroborated these findings, showing a progressive reduction in individual activity over time, which was reflected in the increasing AlarmSum values (Figure S10 in section 4.3 in SI-A).

We applied the previously established decision-making framework (Kizgin et al., 2024) to systematically exclude potential influences of confounding factors on behaviour, such as technical and abiotic parameters. Once a potential impact of the confounding factors had been ruled out, correlations between target compounds and the toxicity parameters of the BEWS were analysed. Non-target chemical screening was also conducted for all instances in which BEWS toxicity thresholds were exceeded, ensuring the detection of possible contributing contaminants. In the following, the insights resulting from the different information available are described for each case. An overview of target and non-target compounds, along with their identification data is provided in Table 1. Due to confidentiality agreements, detailed information about compound names, the MS2 spectra and the specific chemical structures of these compounds cannot be disclosed but the chemical class is provided. Statistical correlation results between the organisms' responses in BEWS and compound concentrations are presented in Table 2.

3.2. A change in daphnia magna behaviour correlates with a target compound

Among the analysed compounds, Compound A, a confirmed herbicide, was detected by the MS2Field and showed the strongest correlation with the *D. magna* Toxicity Index, though the strength was moderate (Pearson $r = 0.46$) to weak (Spearman $\rho = 0.23$) (Fig. 2), particularly

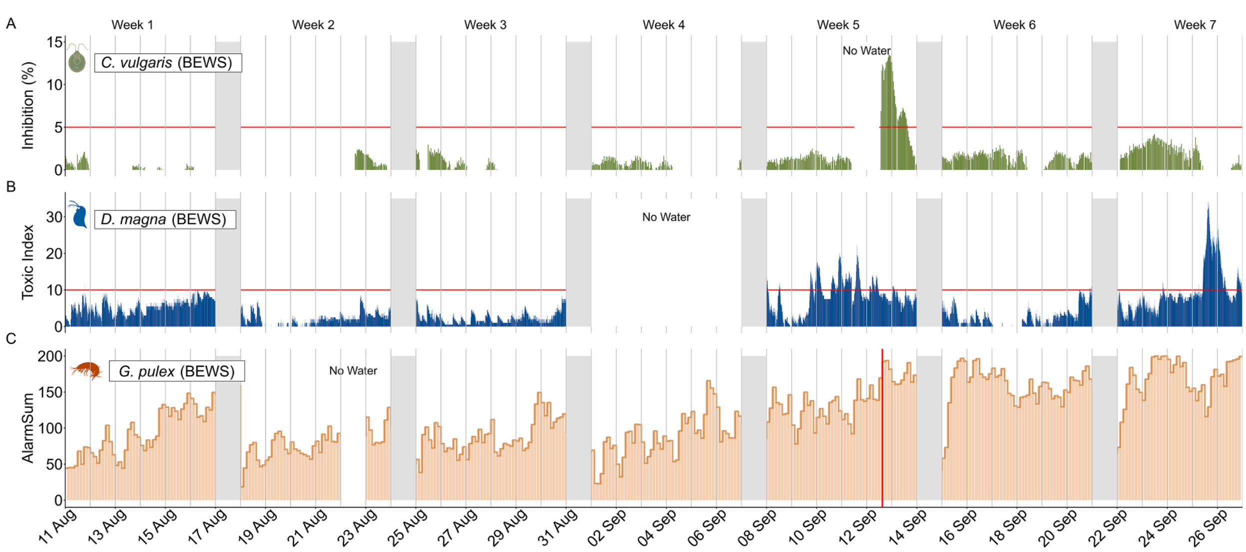

Fig. 1. Toxicity profiles of all BEWS test organisms during the monitoring period of the WWTP effluent. Grey shaded area represents maintenance days with organism replacement. Technical issues occurred during weeks 2, 4, and 5, during which 1–5 days of data were invalid, marked as "no water." (A) Photosynthesis inhibition (% PS inhibition) of *C. vulgaris* (green), with the red horizontal line indicating the toxicity threshold. (B) The Toxic Index of *D. magna* (blue) indicates the deviation from normal behavioural activity in the test chamber ($n = 20$). The red horizontal line marks the toxicity threshold (C) The AlarmSum parameter of *G. pulex* (orange) represents the deviation from normal behavioural activity ($n = 8$). The red vertical line marks a changepoint detected on 2022–09–12 at 15:00.

Table 1
Details on the target and non-target compounds that were detected by the MS2Field from August 11 to September 26. Concentrations of non-target substances were not determined (n.d.).

Compound ID	Target/ Non-target	Chemical Class/ Function	Neutral mass (Da)	Retention time (min)	Identification Confidence (Schymanski Level)	Quality Criteria Applied	Concentration (max) µg/L	Library Match (%)
A	Target	Herbicide (ALS Inhibitor)	~453	11.47	Level 1 (Confirmed structure)	Reference standard (RT, MS2 match, isotopic pattern)	2.03	–
B	Non-target	Aromatic Amine	~122	6.91	Level 2a (Probable structure via library MS2)	Exact mass, RT, MS2 spectral library match	n.d.	91.7
C	Non-target	Aromatic Amine	~146	7.09	Level 2a (Probable structure via library MS2)	Exact mass, RT, MS2 spectral library match	n.d.	86.5
D	Non-target	Nitrogen-containing compound	~135	9.77	Level 4 (Formula assigned)	Exact mass, isotope pattern	n.d.	–
E	Non-target	Nitrogen-containing compound	~106	4.93	Level 4 (Formula assigned)	Exact mass, isotope pattern	n.d.	–
F	Non-target	Nitrogen-containing compound	~146	7.09	Level 4 (Formula assigned)	Exact mass, isotope pattern	n.d.	–
G	Non-target	Nitrogen-containing compound	~122	6.91	Level 4 (Formula assigned)	Exact mass, isotope pattern	n.d.	–
H	Non-target	Nitrogen-containing compound	~107	9.77	Level 4 (Formula assigned)	Exact mass, isotope pattern	n.d.	–

Table 2
Correlations between target and non-target compounds and BEWS responses. Correlation strengths were assessed using both Pearson and Spearman coefficients. Values $\geq$ 0.7 were considered strong, 0.5 – 0.7 moderate, 0.3 – 0.5 weak, and $<$ 0.3 negligible.

Compound ID	BEWS (test organism)	Pearson (r)	Spearman (ρ)	Correlation Strength
A	*DaphTox II (D. magna)*	0.46	0.23	Weak (r), Negligible (ρ)
B	*Algae Toximeter (C. vulgaris)*	0.72	0.45	Strong (r), Moderate (ρ)
C	*Algae Toximeter*	0.76	0.44	Strong (r), Moderate (ρ)
D	*Sensaguard (G. pulex)*	0.58	0.51	Moderate
E	*Sensaguard (G. pulex)*	0.51	0.44	Moderate (r), Weak (ρ)
F	*Sensaguard (G. pulex)*	0.46	0.41	Weak
G	*Sensaguard (G. pulex)*	0.29	0.29	Weak
H	*Sensaguard (G. pulex)*	0.28	0.29	Weak

during the period from September 25 to 26 (Table 2). This compound, originating from an industrial production process, was identified and quantified using a reference standard, with a measured maximum concentration of 2 µg/L in the effluent (Table 1). No other target or non-target compound was detected that correlated with the behaviour of *D. magna* during this time. Notably, Compound A was on the industrial cluster's target list and is regularly monitored.

The 48 h-EC_{50} value for this herbicide in relation to *D. magna* is reported to be 100 000 µg/L, indicating that the detected concentration in the effluent is significantly lower than known levels required to cause acute mortality in *D. magna*. Chevalier et al. (2015) and Villa et al. (2018) already explored that behavioural endpoints are far more sensitive for daphnia than mortality, but there is a lack of information on how much the sensitivity can differ between the endpoints. In the described case the difference factor between behavioural responses and mortality would be four orders in magnitude. Despite the high difference, the strong correlation with the Toxic Index suggests sub-lethal effects on *D. magna* behaviour or physiology, which probably could not be captured by standard mortality endpoints (Altenburger et al. 2019; Bownik, 2020). It is important to note that although HPLC-ESI-HRMS/MS is a powerful tool for identifying contaminants, its effectiveness is constrained by limitations such as detection thresholds, variations in ionization efficiency and the complexity of sample matrices. Consequently, the absence of other detected peaks does not rule out the possible influence of undetected compounds and potential mixture toxicity on the observed behavioural responses.

3.3. *Elevated algae toxicity correlates with unknown aromatic amines*

During the occurrence of elevated photosynthesis inhibition observed from September 12 to 14 with the Algae Toximeter, no significant correlations were found with target compounds. However, the application of NTS revealed two previously unidentified compounds (Fig. 3), whose intensity time profiles strongly aligned with the photosynthesis inhibition profile of *C. vulgaris* ($r = 0.72/\rho = 0.45$ and $r = 0.76/\rho = 0.44$, respectively), indicating a robust and moderately monotonic relationship (Table 2). The integration of BEWS, NTS and advanced data analysis techniques proved to be essential for the efficient localization of these two compounds and the accurate determination of their time profiles. Without this combined approach, identifying potentially toxic compounds within such a complex dataset would have been far more difficult and time-consuming (Niarchos et al., 2024; Vosough et al., 2024).These two compounds were identified as aromatic amines and thus further referred to as Compound B and Compound C (Table 1). The retention time (RT) and molecular formulas of these compounds were further confirmed through spectral library alignment, achieving identification level 2a according to Schymanski et al. (2014). Compound B was identified with a confidence level of 92 %, while Compound C had a slightly lower identification confidence of 85 % according to library spectrum match using mzCloud. Since no reference standards for these compounds were available, confirmation of the structures and quantification could not be performed at this stage. Such a situation is often the case for chemical compounds that are not parent compounds or targeted production compounds.

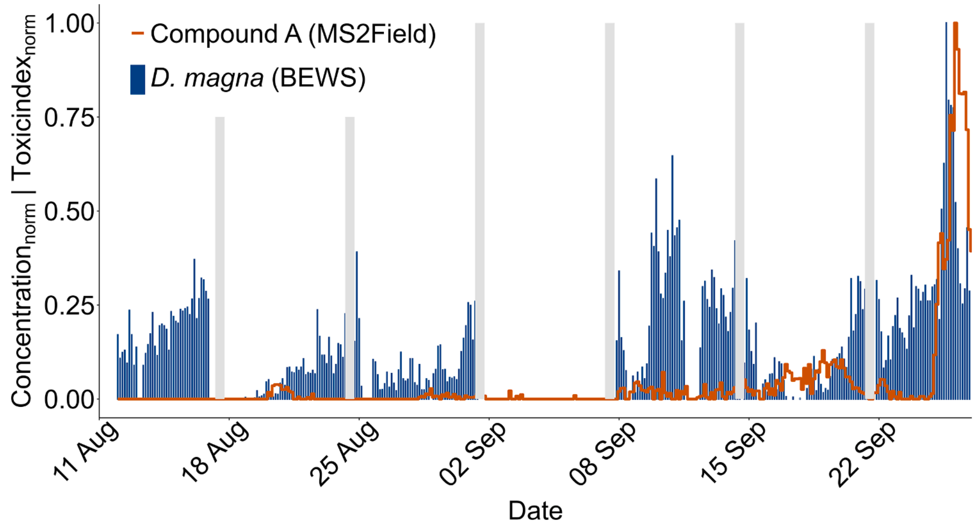

Fig. 2. Time series of normalized concentrations of Compound A (orange line, MS2Field) and the normalized Toxic Index derived from *Daphnia magna* (blue bars, BEWS) from August 11 to September 26. The grey shaded areas indicate maintenance periods involving organism exchange.

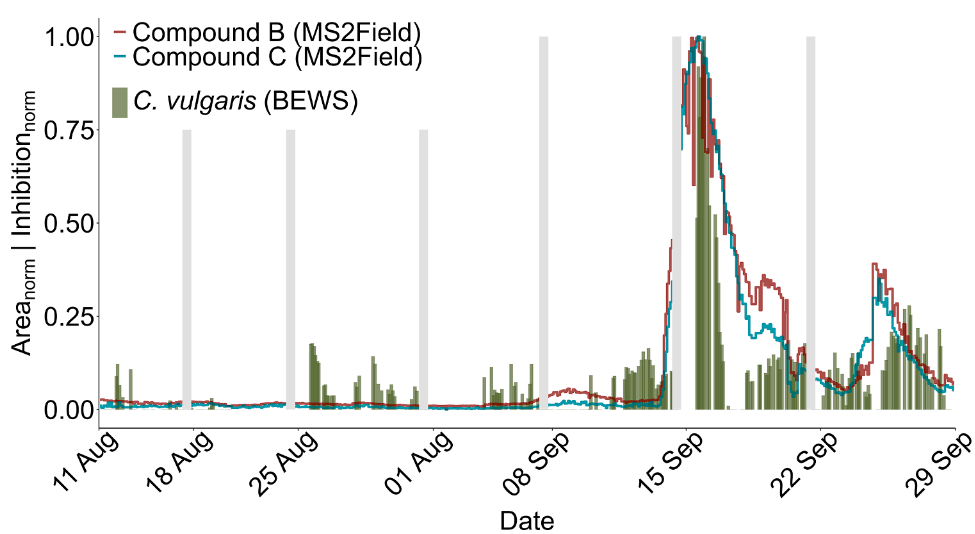

Fig. 3. Time series of normalized area intensity of Compound B (red line, MS2Field), Compound C (blue line, MS2Field) and the normalized inhibition derived from *Chlorella vulgaris* (green bars, BEWS) from August 11 to September 26. The grey shaded areas indicate maintenance periods involving organism exchange.

Ecotoxicological data for these compounds, particularly concerning their effects on daphnids, gammarids, or algae, are currently absent from the literature. However, their molecular formulas and mass-to-charge ratios suggest their role as specific intermediates in the production of complex chemical compounds, such as dyes, pharmaceuticals, and agricultural chemicals. The exact timing of the occurrence of specific masses, combined with their inferred ecotoxic potential, offers manufacturers valuable insights into which chemical processes were active during particular phases of their production cycles. However, with a growing number of production lines within industrial clusters, implementing this approach becomes increasingly complex. Depending on the level of information available, further chemical measurements can then be carried out at the suspected processes. Alternatively, a direct assessment can be made of which adjustments are suitable for reducing the emission of these compounds.

3.4. A shift in gammarus pulex behaviour indicates the presence of non-target compounds

For *D. magna*, the first exceedance of the toxicity threshold occurred during the fifth week of monitoring (Fig. 1A). In the same week, a shift in the circadian rhythm of *G. pulex* was observed and a changepoint was detected (Fig. 1C). The circadian rhythm, a 24-hour autonomous biological clock, can serve as an indicator of metabolic changes and stress, both of which can significantly affect monitoring outcomes (Yang et al., 2018). Zhao et al. (2020) emphasized the importance of circadian rhythms in the process of organisms' ecological responses, and Kizgin et al. (2023) demonstrated that chemical exposure can influence behavioural activity and disrupt circadian rhythm regulation in *G. pulex*. Based on these findings, a more in-depth analysis was performed to evaluate which chemicals were present in the wastewater during this period.

To narrow down potential causes for this behavioural change, the target and NTS data from September 7 to 17 were examined. This analysis revealed no target compounds that correlated with the behavioural responses of *D. magna* and *G. pulex*, but the presence of five non-target compounds that coincided with the behavioural shift and correlated moderately ($r = 0.39$–0.51, $\rho = 0.41$–0.51) with the increase in the AlarmSum of *G. pulex* were found during this period (Fig. 4 and Table 2). It was not possible to clearly identify these compounds due to missing

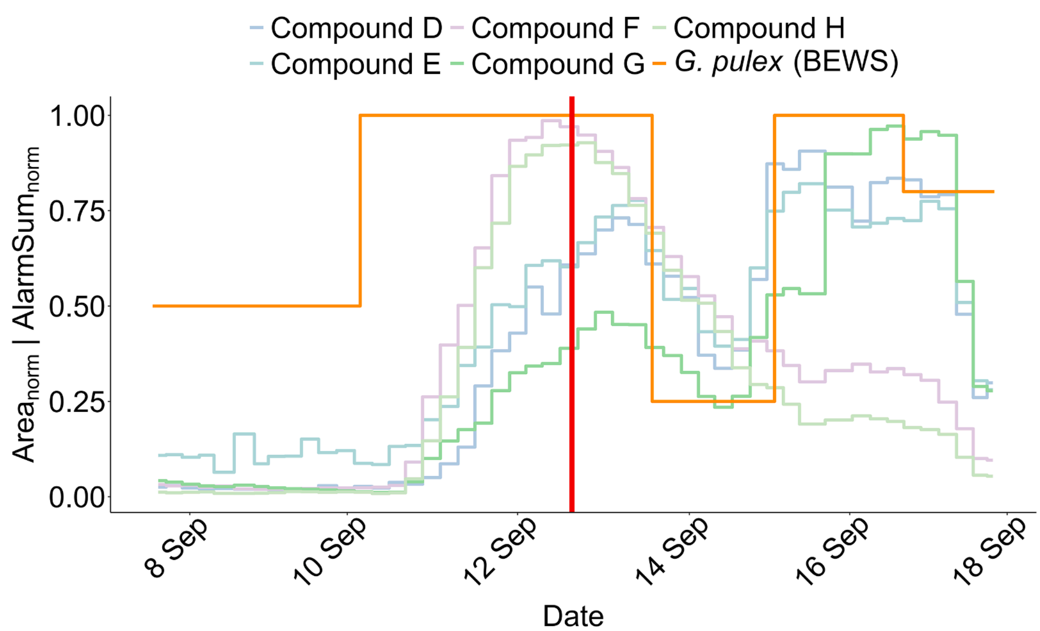

Fig. 4. Normalized area of the NTS compounds detected with the MS2Field (Compounds C-G, green to blue lines) that occurred at the same time with the increase of the AlarmSum parameter of *G. pulex* (in orange). The vertical red line indicates the changepoint of the *G. pulex* behaviour.

spectral library matches. We achieved a level 4 identification based on Schymanski et al. (2014) as molecular formula could be assigned using the exact mass and isotope information (Table 1). Tracing back the origin of these compounds remains a significant challenge when only molecular formula and masses are available.

While time-resolved monitoring data provide insights into compound dynamics, relying solely on correlating these patterns with production calendars or maintenance schedules of complex industrial clusters remains a challenge without the aid of detailed LC-MS identification. Clear and unambiguous identification is essential if industry is to make meaningful progress in dealing with such unknown compounds.

The correlation of chemical data with the *G. pulex* behavioural signal (AlarmSum) presented particular challenges. The signal is shaped by the organism's pronounced circadian activity rhythm, resulting in non-linear and cyclic fluctuations that make direct associations with chemical exposure patterns difficult. This complexity is evident in the time series data (Fig. 4), where fluctuations in AlarmSum generally align with rising compound concentrations, decline with decreasing levels, and show variable responses to re-exposure (e.g., in the cases of compounds F and H). These non-linear dynamics highlight the limitations of relying solely on Pearson correlation, which assumes linearity. To address this, the Spearman correlation was additionally applied, as it better captures monotonic relationships and is more robust to cyclic or non-normally distributed data, such as the behavioural responses of *G.pulex*. The fact that Spearman and Pearson correlations yielded converging results for several compounds (e.g., compound D: $r = 0.58$ / $\rho=0.51$) strengthens the evidence for a potential association, despite the system's inherent complexity (Table 2).

Amphipods such as *G. pulex* remain highly relevant for BEWS applications due to their ecological significance and sensitivity to specific pollutant classes, including neonicotinoids (Ashauer et al., 2011) and pyrethroids (Gerhardt, 2011; Deanovic et al. 2013). Their role as benthic detritivores and their position in the aquatic food web (Kunz et al., 2010) justify their continued use, even if data interpretation requires greater caution and methodological refinement.

To enhance the interpretation of behavioural data from amphipods, it is essential to evaluate the connections between compound-induced effects using advanced statistical tools such as multivariate changepoint analysis (Kizgin et al., 2023). This approach assists in overcoming the limitations encountered with linear correlation models or relying solely on manufacturers' alarm algorithms and suggests that incorporating sophisticated analytical methods can unlock more nuanced insights into environmental contaminants effects on amphipod behaviour. Ruck et al. (2023) provides additional support for the application of amphipods in BEWS. Their study underscores the potential of *G. fossarum* as a sentinel organism for the detection of micropollutant peaks in WWTP effluents through behaviour-based monitoring. Their research demonstrates that controlled minimal activity states in *G. fossarum* enhance the sensitivity and reproducibility of avoidance behaviour signals in response to contaminants. The rapid and distinct behavioural responses enabled the identification of transient contamination events, suggesting a broader applicability for monitoring complex effluents.

3.5. *Online monitoring tools compared to laboratory analysis of composite samples*

3.5.1. *Comparison between online and laboratory chemical analysis*

To compare the MS2Field online measurements with conventional chemical analysis, results from weekly composite samples were used. These included analyses conducted by the industrial site after a maximum of one week of storage at 2–8 °C, and by Eawag after six months of storage at –20 °C, for three industrial target compounds: Compound A, Compound I, and Compound J.

The MS2Field detected and quantified Compound A at a maximum concentration of 2 μg/L (Table 1). In contrast, the composite samples analysed by the industry reported a concentration of 1.2 μg/L, representing a 40 % reduction, while the samples analysed by Eawag revealed a concentration of 0.9 μg/L, indicating a 55 % loss relative to the maximum concentration. Decrease of concentration in the composite samples was partly visible in the quantification of Compound I and J presented in section 8 in the SI-A.

The discrepancy in results between the different analyses can be attributed to the dilution effect inherent in composite sampling. Additionally, the storage of these samples may lead to compound degradation. This degradation process is influenced by various factors. Organic compounds can deteriorate even under freezing conditions, with the rate of degradation depending on the compound's susceptibility to hydrolysis, oxidation, or other chemical reactions. The wastewater matrix itself plays a critical role, as interactions with other compounds can accelerate these processes (Petrović et al., 2005). Such degradation likely contributed to the partially lower concentrations reported by the lab analysis conducted six months later.

Given these challenges, our findings highlight the importance of analysing wastewater samples immediately after collection to ensure data accuracy and reliability. When immediate analysis is not possible, strict storage protocols are essential to preserve sample integrity and avoid degradation of compounds of interest as much as feasible (Köck-Schulmeyer et al., 2013).

3.5.2. Comparison of organism responses in BEWS and laboratory bioassays

To compare the responses of organisms in the BEWS with the responses of organisms in laboratory bioassays using composite samples, effects of the wastewater on algal photosynthesis in the Algae Toximeter were compared with effects on algal and macrophyte growth in the bioassays.

The elevated concentrations of Compound A between August 25 and September 26 did not lead to effects on the photosynthetic activity of the algae *C. vulgaris* in the Algae Toximeter, whereas a strong correlation between elevated concentrations of this compound in the composite samples and growth inhibition of the aquatic macrophyte L. *minor* ($r = 0.89$) was observed (Table 2). This suggests that Compound A was likely the primary factor driving the observed growth inhibition. Compound A belongs to herbicides, that act as acetolactate synthase (ALS) inhibitors (Herbicide Handbook, 2007), disrupting the biosynthesis of essential amino acids that are critical for plant growth. Inhibition of ALS impairs cell division, making Compound A predominantly a growth inhibitor. In the literature, toxicity values for growth inhibition tests revealed a concentration of 0.65 μg/L (7 d-EC_{50}) for *Lemna* species, which is within the range of the measured effluent concentrations in the online and in the composite samples. These results support the observed growth inhibition of L. *minor* in the bioassay, further supporting its high sensitivity to ALS inhibitors.

In contrast, the observed growth inhibition in the laboratory bioassay with *R. subcapitata* was relatively minor ($r = 0.22$). The actual growth inhibition for *R. subcapitata* remained weak compared to the clear inhibition observed for L. *minor*, supporting the conclusion that *R. subcapitata* is less sensitive to ALS inhibitors. This aligns with previous studies showing that sensitivity to ALS inhibitors varies significantly by species and mode of action (Ohnuki et al., 2016). While vascular plants like L. *minor* exhibit high sensitivity to ALS inhibitors, microalgae such as *R. subcapitata* are less affected because ALS inhibitors primarily target biosynthetic pathways specific to vascular plants (Ueda and Nagai, 2021). The literature toxicity values of 90 000 μg/L (96 h-EC_{50}) for *R. subcapitata* suggests a lower sensitivity towards Compound A compared to L. *minor*. Similarly, the lack of effect on *C. vulgaris* in the Algae Toximeter is consistent with the classification of Compound A as a growth inhibitor rather than a direct inhibitor of photosynthesis. Herbicides that directly inhibit photosystem II (e.g., triazines and uracils) act via a completely different mechanism. Currently, no BEWS assess algae growth inhibition directly. This endpoint requires extended incubation times, often spanning several days, to allow sufficient expression of toxic effects on growth. Such time demands present significant challenges to integrating growth inhibition assays into real-time biomonitoring systems. While photosynthesis measurements, such as those used in the Algae Toximeter, offer rapid assessments of acute toxicity, they may not capture the chronic growth-inhibiting effects that are critical for evaluating herbicides like Compound A.

In conclusion, selecting the right organisms for bioassays or BEWS and incorporating a battery of multiple assays or systems, is fundamental and recommended for evaluating the varied effects of pollutants like Compound A that can occur in complex industrial wastewater (Bae and Park, 2014; Kizgin et al., 2023; Xu et al., 2020).

3.6. Contribution of BEWS and hplc-esi-hrms/ms to industrial wastewater monitoring

Our study demonstrates a new strategy that leverages technological advances, particularly online monitoring tools, to support the industrial efforts to reduce emissions. The combination of BEWS and automated HPLC-ESI-HRMS/MS using the MS2Field enhanced the identification of potentially harmful compounds in industrial effluent by providing real-time, high-resolution data. BEWS, traditionally used in drinking and surface water monitoring but rarely in the wastewater sector, proved valuable for industrial wastewater applications, providing a rapid alternative to conventionally used bioassays (Bownik and Wlodkowic, 2021; Sepman et al., 2023). While previously successful in detecting pesticides within karst groundwater (Schorr et al., 2024) and target and non-target compounds in municipal wastewater (Kizgin et al., 2024), the integration of the MS2Field further demonstrated the power of high-resolution mass spectrometry to detect unexpected and trace compounds in industrial wastewater. A key aspect of the MS2Field was its ability to provide based on time profiles tentative identifications of detected compounds with varying confidence levels. It is important to note that even without full structural elucidation, tracking the presence of certain compounds over time allows for the identification of toxicologically relevant compounds which might otherwise be missed by traditional methods. To support the interpretation of these high-frequency datasets, a structured workflow was applied: time series from BEWS and MS2Field were aligned and pre-processed, including smoothing, baseline correction, and normalization where necessary. Changepoint detection using the PELT algorithm (Killick et al., 2012) was applied to identify significant signal shifts in BEWS endpoints, and Pearson and Spearman correlation analyses were used to assess associations between BEWS responses and chemical features. This integrative workflow enabled the targeted evaluation of candidate toxicity drivers in complex industrial effluent under real-world conditions.

3.7. Collaboration with WWTP operators and stakeholders

A major key success factor in this study was the collaboration between industrial WWTP operators and environmental researchers. By fostering open communication and collaboration, ways can be found to identify harmful compounds, search for them in the different production streams, facilitate optimization of existing processes and thus enable reduction of emissions. The availability of time-resolved data from tools like BEWS and HPLC-ESI-HRMS/MS enhances the precision of monitoring efforts by pinpointing periods of peak pollutant discharge. These observations can be cross-referenced with production schedules and operational data, enabling industries to trace pollutants back to specific activities or processes. For instance, a spike in toxic emissions during a particular production phase may signal inefficiencies or the need for process optimization. By identifying such correlations, industries can not only reduce their environmental footprint but also improve resource efficiency and cost-effectiveness. The active involvement of industrial stakeholders is crucial to ensure that technological advancements, such as BEWS and HPLC-ESI-HRMS/MS, are practically implemented in a way that meets real-world operational constraints. Although detailed source-tracking was beyond the scope of this study, the identified toxicity events and their associated chemical profiles were shared with the industrial partner and subsequently used to refine routine suspect screening within the industrial cluster. This supported the proactive identification and management of potential pollution sources.

3.8. Barriers to wider adoption and future outlook

This study illustrates how chemical monitoring tools can be combined to provide reliable data on what triggered the BEWS signals. Despite these benefits, the widespread adoption of online monitoring tools faces certain challenges. A significant barrier is the need for technical equipment and particularly expertise, as the vast amount of data generated requires sophisticated frameworks for analysis and interpretation. Successful application of these technologies demands not only expertise in HPLC-ESI-HRMS/MS data processing, but also an understanding of biomonitoring and sensor technology. The application of continuous monitoring workflows requires not just sufficient time and resources, but also strategic decision making, as both the reliability of BEWS signals and the complexity of the chemical data must be carefully managed to effectively identify and prioritise toxicity drivers in industrial effluents.

A key challenge in linking BEWS responses to specific toxicants in industrial wastewater is the inherent complexity of effluent matrices, which often contain mixtures of known and unknown substances. These mixtures can lead to additive, synergistic, or antagonistic effects, complicating the interpretation of biological responses and the identification of causal toxicants (Altenburger et al. 2019). Furthermore, matrix effects in chemical analysis, such as ion suppression or enhancement, can obscure or distort the detection of relevant compounds. In this study, we mitigated these challenges using isotopically labelled internal standards (ILIS) for chemical quantification, a structured decision-making framework to assess confounding factors and time-series correlation analyses to prioritize potential toxicity drivers. However, we acknowledge that these methods alone do not provide definitive proof of causality. To overcome this limitation, a combination of toxicological assessments—such as comparing observed effect concentrations with available EC_{50} values for identified compounds—and targeted verification experiments with BEWS (e.g., spike tests in effluent samples) can be employed to substantiate the role of suspected toxicants. This strategy was successfully demonstrated in our previous study (Kizgin et al., 2024), where spike tests validated the causative link between chemical signals and observed biological effects.

For more complex mixtures or unknowns, more in-depth methods such as Effect-Directed Analysis (EDA) can be valuable. EDA combines bioassays, sample fractionation, and chemical analysis to identify the specific chemicals responsible for the observed effects (Altenburger et al., 2019; Liu et al. 2024). Recently, Niarchos et al. (2024) proposed an effect-based early warning approach as a proactive tool for identifying hazardous chemicals in environmental matrices. A key element of their framework is the systematic use of bioassays to identify adverse biological effects, paired with high-resolution non-target screening (NTS) to pinpoint bioactive compounds that may be overlooked by conventional monitoring. The authors also emphasize the need for standardized workflows and greater automation in sampling and analysis to improve timeliness and scalability. An alternative approach is virtual EDA (vEDA), which integrates bioassay results with chemical data using statistical models to prioritize toxicants without physical fractionation (Alvarez-Mora et al., 2024). While vEDA offers faster and more scalable analysis, it depends on consistent biological effect data and in-depth understanding of statistical modelling (Zweigl et al., 2025). The parallel use of online monitoring tools could further streamline effect-based workflows by avoiding both labour-intensive fractionation and the complex data analysis required in conventional EDA, instead leveraging time-series data to detect emerging toxicity patterns.

While BEWS technologies are relatively cost-effective, modular, and require only moderate maintenance, their implementation can already support broader industrial deployment. By using biological signals to prioritize timeframes for non-target analysis, the amount of mass spectrometric data requiring in-depth processing can be significantly reduced, minimizing computational workload, analyst time, and data storage needs — a clear advantage over continuous full-spectrum analysis of entire datasets (Minkus et al. 2022). By leveraging BEWS signals to target only selected timeframes with likely toxicological relevance, the number of samples requiring full NTS was reduced to three prioritized time windows within the seven-week monitoring period. This resulted in an estimated cost reduction of over 70 % compared to continuous screening. This targeted approach illustrates not only a scientific advantage in detecting relevant toxicity drivers, but also a practical economic benefit. Nevertheless, online high-resolution mass spectrometry (HRMS) systems such as MS2Field, though powerful for real-time chemical profiling, still involve higher investment costs, increased technical oversight, and greater data processing demands. As reflected in the weekly operational requirements of each system, each BEWS requires about 2–3 hours of hands-on time and moderate infrastructure, while MS2Field requires greater analytical oversight and data management, despite the absence of test organism maintenance (see Table S12 in SI-A). However, ongoing advances in automation, miniaturization, and machine learning (ML) and AI-assisted analytics are expected to significantly reduce operational barriers and enhance the scalability of integrated monitoring systems (Vosough et al. 2024). Although this study's monitoring campaign was limited to a seven-week period, sufficient to demonstrate feasibility and capture several toxicity events, longer-term deployments spanning one to two years could provide more comprehensive insights across seasonal and operational variations.

The creation of multispecies BEWS (Gerhardt, 2020; Ruck et al., 2023) and user-friendly interfaces and standardized protocols could further facilitate the implementation of these technologies, helping to bridge the gap to address sustainable industrial monitoring practices. Although MS2Field is currently restricted to research applications, industries can already benefit from combining BEWS with conventional laboratory-based or stationary chemical analysis of sufficient resolution to attribute BEWS signals to toxic pollutants in the effluent. Incorporating these methodologies into industrial monitoring strategies not only enhances the reliability of BEWS but also aligns with broader environmental protection frameworks, such as the EU Water Framework Directive and the EU Green deal, which aim to ensure early detecting and preventing the release of new pollutants into water systems by source identification and control (Carere et al., 2021; van Dijk et al., 2021).

4. Conclusions

Detecting peak concentrations of environmentally relevant compounds in industrial wastewater using traditional monitoring methods is challenging due to highly variable and complex production patterns. This study highlights the practical potential and applicability of combining BEWS and online HRMS monitoring for industrial wastewater surveillance. It provides a role model for how such integrated monitoring approaches can be successfully applied in complex industrial settings:

- The integration of BEWS and MS2Field was successfully implemented in a routine industrial setting. This allowed the detection of short-term toxicity events that would likely have gone unnoticed using standard weekly sampling approaches.
- Comparative analysis with conventional monitoring methods, including bioassays and targeted analysis of weekly composite samples, confirmed overall trends. However, the online approach provided superior temporal resolution, captured daily fluctuations, and avoided dilution and degradation effects commonly associated with sample storage.
- Using changepoint analysis and Pearson and Spearman correlation analyses, the approach enabled prioritization of candidate toxicants associated with BEWS responses. While definitive causality could not be established, the framework provides a robust screening tool for narrowing down toxicity drivers in complex effluents.

Together, these findings confirm the potential of integrated online monitoring to enhance industrial wastewater surveillance. The strategy not only improves responsiveness and interpretability but also supports more proactive and targeted environmental protection measures, contributing to the long-term protection of aquatic ecosystems.

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CRediT authorship contribution statement

Ali Kizgin: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Michelle Salvisberg:** Visualization, Validation, Data curation, Conceptualization. **Heinz Singer:** Writing – review & editing, Resources, Methodology, Conceptualization. **Sergio Santiago:** Investigation, Methodology, Writing – review & editing. **Juliane Hollender:** Writing – review & editing, Supervision. **Eberhard Morgenroth:** Writing – review & editing, Supervision. **Cornelia Kienle:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Conceptualization. **Miriam Langer:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

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Data availability

The data that has been used is confidential.

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