Application Note

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Analysis of Wastewater Effluent Samples to Identify Toxic Chemicals Using the High-Resolution Agilent 7250 GC/Q-TOF

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Abstract

This study used a workflow for broad scope suspect screening to identify toxic chemicals in wastewater effluents. The comprehensive approach combined targeted and untargeted methods using a high-resolution accurate mass Agilent 7250 GC/Q-TOF in multiple ionization modes, the GC/Q-TOF screening workflow in Agilent MassHunter Quantitative Analysis software 10.1, and the GC/Q-TOF accurate mass library of pesticides and environmental contaminants.

Introduction

Identification of toxic chemicals in wastewater effluents is a key step towards improving environmental water quality of downstream ecosystems. Conventional targeted analysis approaches are often not sufficient to identify the source of toxicity due to their limited scope. Comprehensive screening, often with nontargeted analysis, can help address this issue and lead to a deeper understanding of the possible cause of toxicity. To comprehensively characterize chemicals in environmental samples, both LC and GC separation techniques coupled to high-resolution mass spectrometry (HRMS) are required. Otherwise, if the focus is only on the LC/MS, many nonpolar and volatile compounds present in these samples can be missed. Accurate mass as well as soft ionization with a GC/HRMS can offer important benefits for identification of unknowns or verification of tentative hits from the NIST library.

This study combined broad scope suspect screening and a nontargeted approach for pesticides and environmental pollutant detection using high-resolution GC/Q-TOF analysis to identify toxic chemicals in wastewater effluent samples.

Experimental

Samples

The wastewater effluent samples (94940, 94941, 94943, and 94944) were collected on days 1, 2, 4, and 5 of a five-day series in duplicates. The first two samples displayed acute toxicity towards *Ceriodaphnia dubia*, as shown by whole effluent toxicity (WET) testing.

Samples were filtered through a GF/F filter (0.45 µm) and passed over a hydrophilic reversed-phase solid phase extraction cartridge. Dried cartridges were eluted with ethyl acetate and methanol. Dried filters were extracted in a sonicating bath with hexane/acetone 1:1. Both extracts were combined and spiked with dibromooctafluorobisphenol (DBOFB) as an internal standard.

The samples were analyzed using the high-resolution 7250 GC/Q-TOF coupled to an Agilent 8890 GC with a 15 × 15 m midcolumn backflush configuration (Figure 1). A 20 minute retention time locked (RTL) method (locked to chlorpyrifos-methyl at an RT of 9.143 minutes) was used to ensure RT consistency with the GC/Q-TOF accurate mass library of pesticides and environmental pollutants. Backflush within the method helped maintain consistent RTs, avoid carryover, extend column lifetime, and reduce source contamination.

The samples were screened for contaminants in electron ionization (EI) and negative CI (NCI) modes to ensure best analytical sensitivity for pyrethroids and other halogenated compounds often seen in wastewater effluents. Positive CI (PCI) was later used to assist compound identification since it favors the formation of molecular ion adducts. Table 1 describes the conditions.

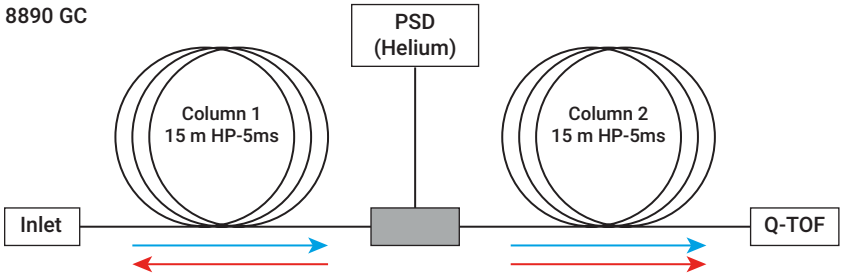


Figure 1. Midcolumn backflush configuration. The helium flowpath during the backflushing at the end of the run is depicted by red arrows. The pressure at the purged union is increased while the pressure at the inlet drops. This results in reversing the flow on the first column and allows high boiling point compounds to be removed through the split vent. The pneumatic switching device (PSD) was an Agilent 8890 GC pneumatic control module, which provided backflush capability with significantly reduced carrier gas consumption.

Table 1. GC/Q-TOF acquisition parameters.

GC and MS Conditions	EI	Negative CI	Positive CI
GC	Agilent 8890 GC		
Column	2 × HP-5ms UI, 15 m, 0.25 mm, 0.25 µm		
Inlet	MMI, 4 mm UI liner single taper with wool		
Injection Volume	1 µL		
Injection Mode	Cold splitless		
Inlet Temperature	60 °C for 0.2 minutes; 600 °C/min to 320 °C		
Oven Temperature Program	60 °C for 1 minute; 40 °C/min to 170 °C; 10 °C/min to 310 °C; 3 minutes hold		
Carrier Gas	Helium		
Column 1 Flow	~1.2 mL/min		
Column 2 Flow	~ 1.4 mL/min		
Backflushing Conditions	5 minutes (post run), 310 °C (oven), 50 psi (AUX EPC pressure), 2 psi (inlet pressure)		
Transfer Line Temperature	280 °C		
Mass Range	*m/z* 50 to 650		
Spectral Acquisition Rate	5 Hz		
Quadrupole Temperature	150 °C		
Source Temperature	280 °C	150 °C	280 °C
Electron Energy	70 eV	250 eV	100 eV
Emission Current	5 µA	10 µA	15 µA

Suspect screening workflow

The GC/Q-TOF data, acquired in EI mode, were first processed using the GC/Q-TOF screening workflow available in MassHunter Quantitative Analysis software 10.1. The list of targets was based on the fully curated accurate mass pesticide personal compound database and library (PCDL), containing over 1,000 unique compounds. To increase the data processing speed and quality, the GC/Q-TOF data were converted to the SureMass format before the analysis. The screening method was automatically created from the GC/Q-TOF pesticides and environmental contaminants PCDL using a total of seven of the most specific accurate mass ions along with their ratios from each spectrum. Screening method parameters were set according to the SANTE[1] and FDA[2] guidelines, and included RT window, mass accuracy, and library match score, among others.

Nontargeted identification workflow

In the nontargeted analysis approach, EI data were processed by Unknowns Analysis in MassHunter Quantitative Analysis 10.1. The feature finding step was followed by a NIST17 library search and the differential analysis in Agilent Mass Profiler Professional (MPP) to identify compounds that correlate with higher toxicity of the wastewater effluent samples. To further identify the molecular ion of the unknown compounds from nontargeted screening or compounds that required identity verification, the Fragment Formula Annotation tool of MassHunter Qualitative Analysis 10 was used with both EI and positive chemical ionization (PCI) GC/Q-TOF data.

To use the NCI GC/Q-TOF data with the GC/Q-TOF screening workflow, a more focused (~120 compounds, such as pyrethroids) accurate mass PCDL based on the spectra acquired in NCI mode was created. Then, the NCI data were also processed using the same GC/Q-TOF screening tool.

Results and discussion

Suspect screening in EI mode

The wastewater effluent samples, displaying varying degrees of acute toxicity (0 to 80%) toward *Ceriodaphnia dubia*, were analyzed using high-resolution GC/Q-TOF. A screening workflow available in MassHunter Quantitative Analysis software 10.1 (Figure 2) was used to surveil many important pesticides and environmental pollutants included in the GC/Q-TOF PCDL.

Using this workflow, over 90 compounds were identified in each wastewater effluent extract using the EI GC/Q-TOF PCDL with mass accuracy <5 ppm and library match scores >75. Figure 3 shows an example of the EI screening window as well as the report. The compounds verified automatically in the screening workflow are labeled in green.

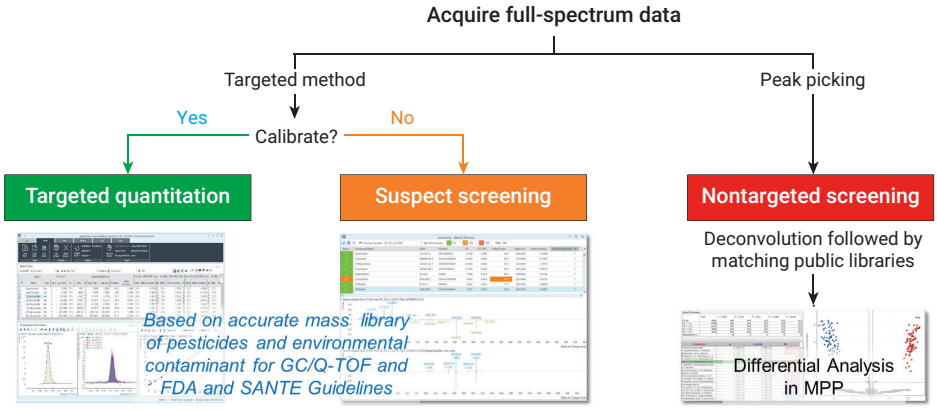


Figure 2. Combined contaminants screening workflow based on a targeted and suspect screening approach using GC/Q-TOF PCDL and nontargeted screening using a NIST library, all performed within Agilent MassHunter Quantitative Analysis 10.1 software.

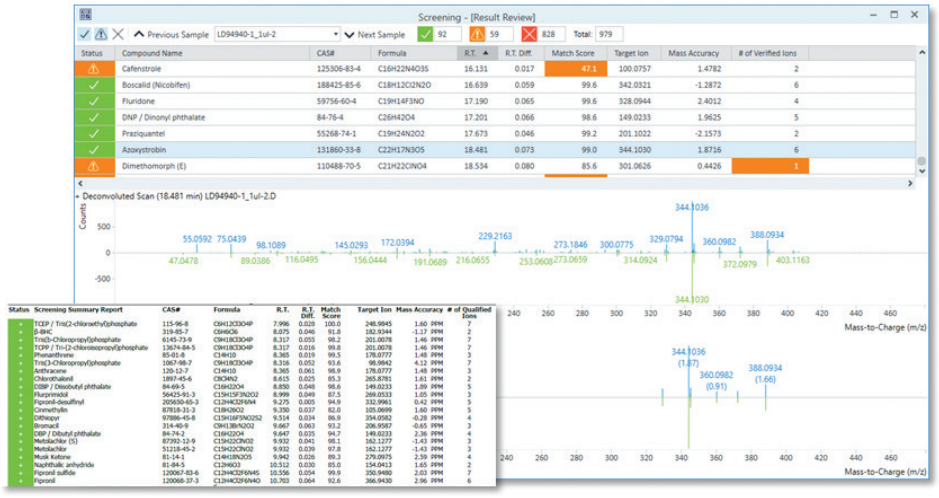


Figure 3. EI PCDL-based screening in Agilent MassHunter Quantitative Analysis software 10.1. Automatically verified compounds are labeled in green. The compounds that need additional review are in orange. A partial report example is shown in the lower corner.

The compounds that need additional manual review are labeled in orange. These tentative hits (highlighted in orange) even with just one failed parameter, such as library match score or the number of verified ions, can be false positives unless they have only a few (and not selective) ions in their spectrum or low response. The GC/Q-TOF suspect screening workflow provides a lot of flexibility, so that the method can be specifically optimized for these challenging compounds.

The compounds identified in GC/Q-TOF screening that demonstrated some difference in the response levels between the samples grouped by mortality % towards *C. dubia* are shown in Table 2, and are potentially those to be investigated as the causative agents for the toxicity.

NCI analysis of wastewater effluent samples

The wastewater effluent extracts previously described were also analyzed in NCI mode to ensure best analytical sensitivity for halogenated contaminants and other compounds with electron-withdrawing groups. NCI data were analyzed using the same GC/Q-TOF screening workflow. A more specific PCDL containing NCI spectra of halogenated nitro compounds and other contaminants with electron capturing groups was created to be able to use the GC/Q-TOF screening approach in NCI. On average, approximately 40 compounds were positively identified in each wastewater effluent using the NCI mode with NCI PCDL. Figure 4 and Table 3 show NCI screener results. Although a few compounds have only one prominent ion or isotopic cluster and may require additional work for confirmation (Figure 4A), most compounds produced enough fragments with methane NCI to be used in the PCDL

Table 2. Selected results from EI suspect screening. The response value is color-coded based on the relative level across the samples. Mass error for the quantifier ion as well as library match score are also displayed.

Sample	80 % Mortality						20 % Mortality						0 % Mortality					
	LD94940-1			LD94940-2			LD94941-1			LD94941-2			LD94943-1			LD94943-2		
Compound Name	Response	Mass Error	Library Match score	Response	Mass Error	Library Match score	Response	Mass Error	Library Match score	Response	Mass Error	Library Match score	Response	Mass Error	Library Match score	Response	Mass Error	Library Match score
TBEP/*Tris*(2-butoxyethyl) Phosphate	2013504	2.8	99.9	1502528	3.9	99.9	1289372	2.5	99.9	1559301	3.8	99.9	787113	3.1	99.9	784473	3.8	99.9
tert-Butylphenyldiphenylphosphate	16799	2.1	92.9	4948	3.2	74.6	2828	1.1	82.5	10468	0.8	91.9	2950	1.3	70.6	2766	0.8	91.9
Chlorantraniliprole	6298	0.2	76.8	5330	2.0	79.4	3572	1.7	63.2	3494	1.8	66.4	3458	1.1	52.4	2710	1.8	66.4
Flurprimidol	16518	1.3	80.4	15240	0.5	76.4	10698	2.6	73.7	12065	2.1	80.2	6038	2.0	74.2	4976	2.1	80.2
Paclobutrazol	16985	0.9	96.8	15763	1.6	98.7	10725	0.9	92.4	12090	2.1	94.9	9106	1.8	79.1	8448	2.1	94.9
TBZ/Thiabendazole	1570235	1.4	99.7	1536170	2.4	99.7	1282402	0.6	99.7	1368732	2.2	99.8	774093	0.6	99.7	675439	2.2	99.8
Azoxystrobin	134463	1.8	99.1	139960	3.0	98.9	109579	1.4	98.9	119004	1.7	98.8	104804	1.7	89.9	94511	1.7	98.8

Table 3. NCI suspect screening results.

Sample	94940-1		94940-2		94941-1		94941-2		94943-1		94943-2	
Compound Name	Response	Library Match Score	Response	Library Match Score	Response	Library Match Score	Response	Library Match Score	Response	Library Match Score	Response	Library Match Score
Deltamethrin	16837	71.4	14202	42.7	6474	83.7	4138	36.3	3253	56.5	4294	36.3
Endosulfan sulfate	3372	98.8	3013	91.9	12182	98.9	11865	99.1	18103	98.8	15859	99.1
Fipronil sulfone	1184481	99.4	989538	99.3	1058932	99.4	898204	99.3	1218463	99.4	1089462	99.3
Chlorfenvinphos	22450	94.7	13196	86.3	16668	94.4	14469	94.7	14757	95.7	12834	94.7
Fipronil	1312800	98.7	1269915	97.7	1255881	97.1	1307988	96.8	1519654	95.8	1350814	96.8
Fipronil-sulfide	201344	99.9	192041	100.0	224062	100.0	218654	100.0	241255	100.0	274001	100.0
Chlorthal-dimethyl	1730		1409	94.1	1468	94.3	1489	96.2	2204	80.0	1807	96.2
Triadimefon	22376	94.1	16547	94.2	19705	96.0	17006	96.4	18710	97.2	16675	96.4
Malathion	474	86.6	249	86.6	0	–	0	–	0	–	0	–
Fipronil-desulfinyl	128886	97.8	111722	97.8	122423	97.8	119001	97.9	164450	97.8	135773	97.9
Chlorothalonil	23789	99.4	12226	99.2	14367	99.0	15765	99.1	14714	99.2	12680	99.1
BHC-beta	36573	88.4	19696	91.7	25594	81.4	19439	84.7	23983	69.4	13527	84.7
Dicloran	30089	92.3	33303	93.2	34005	92.6	39632	93.8	44118	95.1	35911	93.8
Hexachlorobenzene	13573	99.3	10353	99.6	11863	99.3	9934	99.1	14371	98.7	12048	99.1
Trifluralin	10334	86.6	11119	94.1	12089	94.2	11454	92.8	13550	94.5	9293	92.8
2,4-Dinitrotoluene	81406	90.1	91627	89.0	75770	84.4	67256	83.3	43423	91.1	41979	83.3
2,4,6-Trichlorophenol	2551498	92.2	2250861	91.6	2525758	91.5	2544336	91.4	2707308	91.2	2736603	91.4

screening workflow (Figure 4B). Thus, this GC/Q-TOF screening workflow can also be applied to the NCI data whenever there is a corresponding PCDL available.

Using the EI and NCI screening approaches, numerous environmental pollutants were identified in wastewater samples, including many pesticides. However, most of them, including fipronil and fipronil degradation products, were also identified in wastewater samples that exhibited no toxicity (Table 3), and are unlikely to be the source of the observed toxicity.

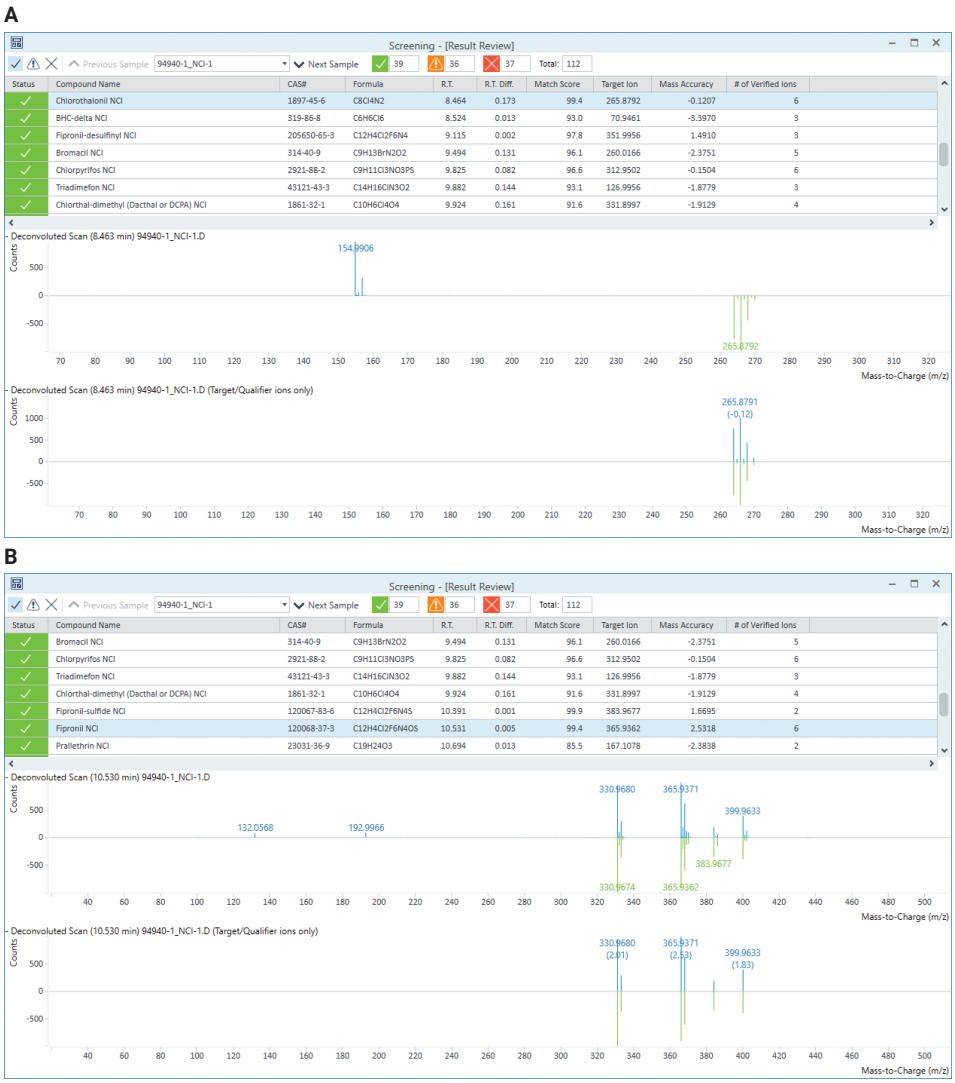


Figure 4. Examples of compounds identified using suspect screening in NCI. The lower mirror plot shows the spectral matching for most specific ions selected by the screening algorithm. A) A hit that has a very simple NCI spectrum. B) An example of a hit with sufficient numbers of ions in the NCI spectrum for confirmation.

Untargeted analysis of wastewater effluent samples

To identify additional contaminants that might be associated with the increased toxicity of the wastewater effluents, the data were processed using an untargeted workflow. This approach involves feature finding in Unknowns Analysis and a NIST 17 library search, followed by differential analysis in MPP to identify compounds with more significant presence in the samples that displayed a higher degree of toxicity (Figure 2).

Identified and unidentified components were exported from Unknowns Analysis as compound exchange format (.CEF) files and imported into MPP. A principle component analysis (PCA) plot showed clear separation into three groups, corresponding to the three wastewater effluents with different toxicities (Figure 5).

The volcano plot of fold change versus statistical significance can assist in visualizing changes in large datasets, and was used in this study to quickly detect differences between 80 and 0% mortality groups (Figure 6). Many compounds were present at significantly higher levels in wastewater effluents that displayed 80% mortality towards *C. dubia* compared to the wastewater effluent extracts with 0% mortality. These compounds can be found at the top right side of the volcano plot, and are colored red.

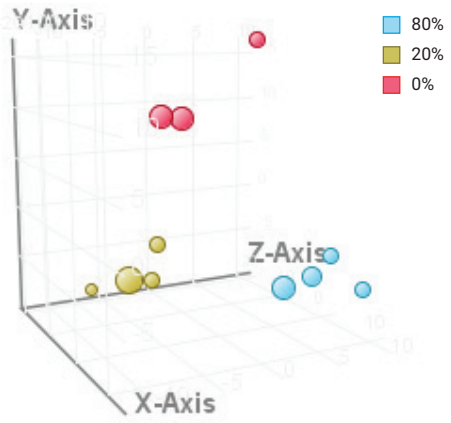


Figure 5. PCA plot indicating clear separation of the wastewater effluent extracts into three groups based on their toxicity.

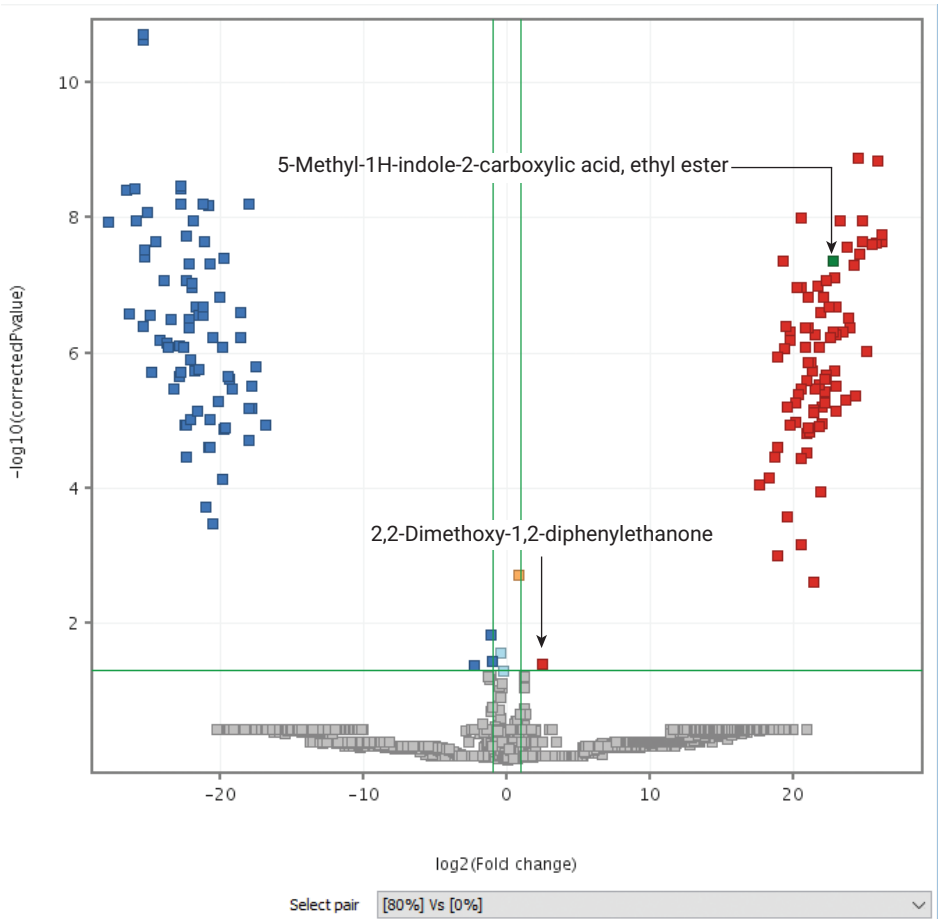


Figure 6. Volcano plot comparing the 80% mortality group against 0%. Compounds labeled with red squares in the top right of the volcano plot are those present at significantly higher levels in the wastewater effluent, characterized with 80% mortality compared to 0% mortality.

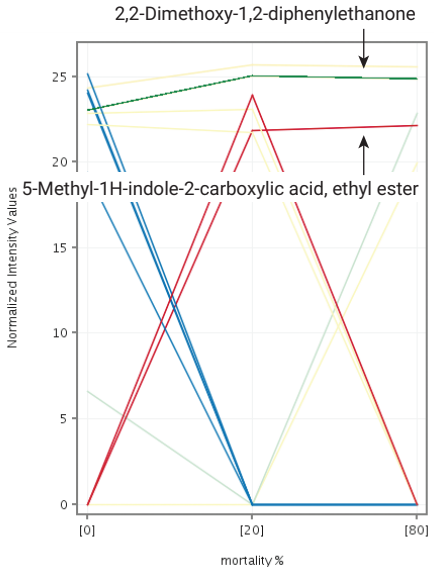
To further find a correlation between the wastewater effluent's toxicity and tentatively identified compounds present in their extracts, a correlation analysis was performed. The fold change (FC) analysis outcome for all three effluent toxicity groups with an FC cutoff of 2 and p <0.05 was used as an entity list (Figure 7A) for correlation analysis. Percent of mortality was chosen as a filtering parameter. The Pearson similarity metric with a cutoff of 0.6 was selected to only display the compounds with strong correlation (Figures 7B and 7C). Note that a couple of tentatively identified compounds in the volcano plot and the correlation analysis (Figures 6 and 7C) were selected for further compound ID confirmation, as discussed in the next section.

Verification of tentative hits and identification of unknowns

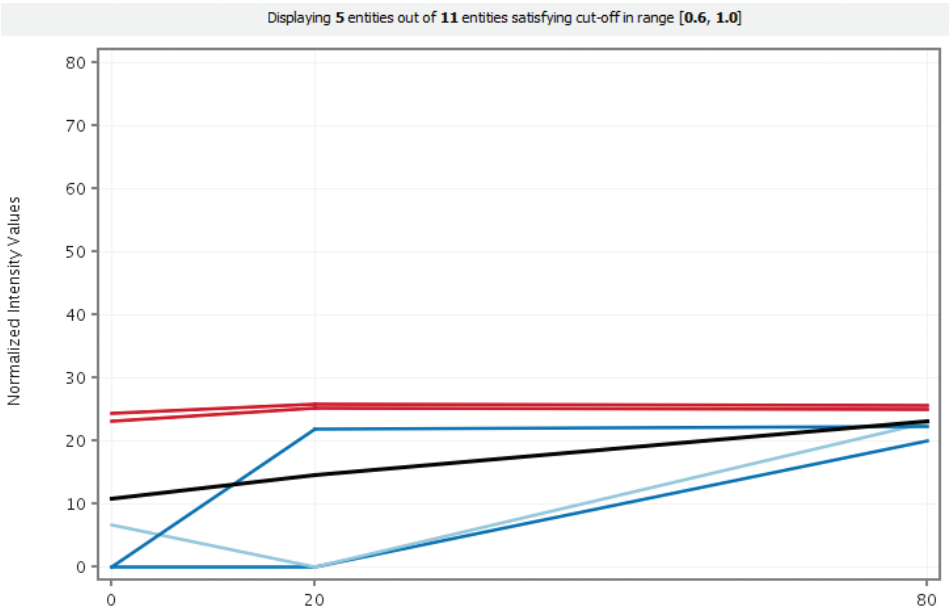
To confirm the identity of the compounds that correlated with the wastewater effluent's toxicity, ExactMass (a fragment formula annotation) feature of Unknowns Analysis was used (Figures 8A and 8B). A compound tentatively identified using the NIST 17.L library as 2,2-dimethoxy-1,2-diphenylethanone with a library match score of 85.9 exhibited a small mass error across all the fragment ions when a tentative hit's molecular formula was considered (Figure 8A). The second compound of interest was misidentified, as the fragments' *m/z* did not match the molecular formula of the tentative hit (Figure 8B). Since the EI spectrum of this compound did not provide any detectable molecular ion, PCI was used to help identify the molecular ion and, together with the Fragment Formula Annotation tool of MassHunter Qualitative Analysis, propose a molecular formula for this unknown (Figure 8C). The PubChem database suggested a structure for this compound corresponding to acetyl triethyl citrate (Figure 8C) as the most relevant for this compound formula.[3]

A

Compound	FC ([20] vs [0])	FC ([80] vs [0])
5-Methyl-1H-indole-2-...	3827410.25	4661339.50
Tributyl acetylcitrate	-1.00	1009510.69
Triisobutylaluminum	-95.22	79011.20
Ethanone, 2,2-dimethox...	4.11	3.68
Tolbutamide	2.55	2.35
Bufa-20,22-dienolide, 1...	16059207.00	-1.00
Ethylphosphonic acid	-667366.50	-667366.50
2-(2-tert-Butyldimethyls...	-1.34	-4735411.50
Myristyl myristate	1.20	-7482716.50
Tricyclo[3.3.3.0(1,5)]un...	-17029808.00	-17029808.00
5H-Pyrrolo(3,2-d)pyrimi...	-19829464.00	-19829464.00
Dendroban-12-one, 10...	-37046200.00	-37046200.00



B



C

Entities | Attributes

Compound	Similarity	Alignment Value	Annotations	CAS Number	ChEBI ID	Compound Na...
Tributyl acetylcit...	0.97073	Tributyl acetylcit...	Tributyl acetylcit...	77-90-7		Tributyl acetylcit...
Ethanone, 2,2-d...	0.64071	Ethanone, 2,2-d...	Ethanone, 2,2-d...	24650-42-8		Ethanone, 2,2-...
Triisobutylalumi...	0.86491	Triisobutylalumi...	Triisobutylalumi...	100-99-2		Triisobutylalumi...
Tolbutamide	0.63448	Tolbutamide	Tolbutamide [C...	64-77-7		Tolbutamide
5-Methyl-1H-in...	0.70139	5-Methyl-1H-in...	5-Methyl-1H-in...	1000318-48-3		5-Methyl-1H-in...

Figure 7. Correlation analysis in Agilent MPP. A) Fold change (FC) analysis across three wastewater effluent samples; B) filter on parameters output; C) compound list as a result of the correlation analysis when using a similarity cutoff of 0.6.

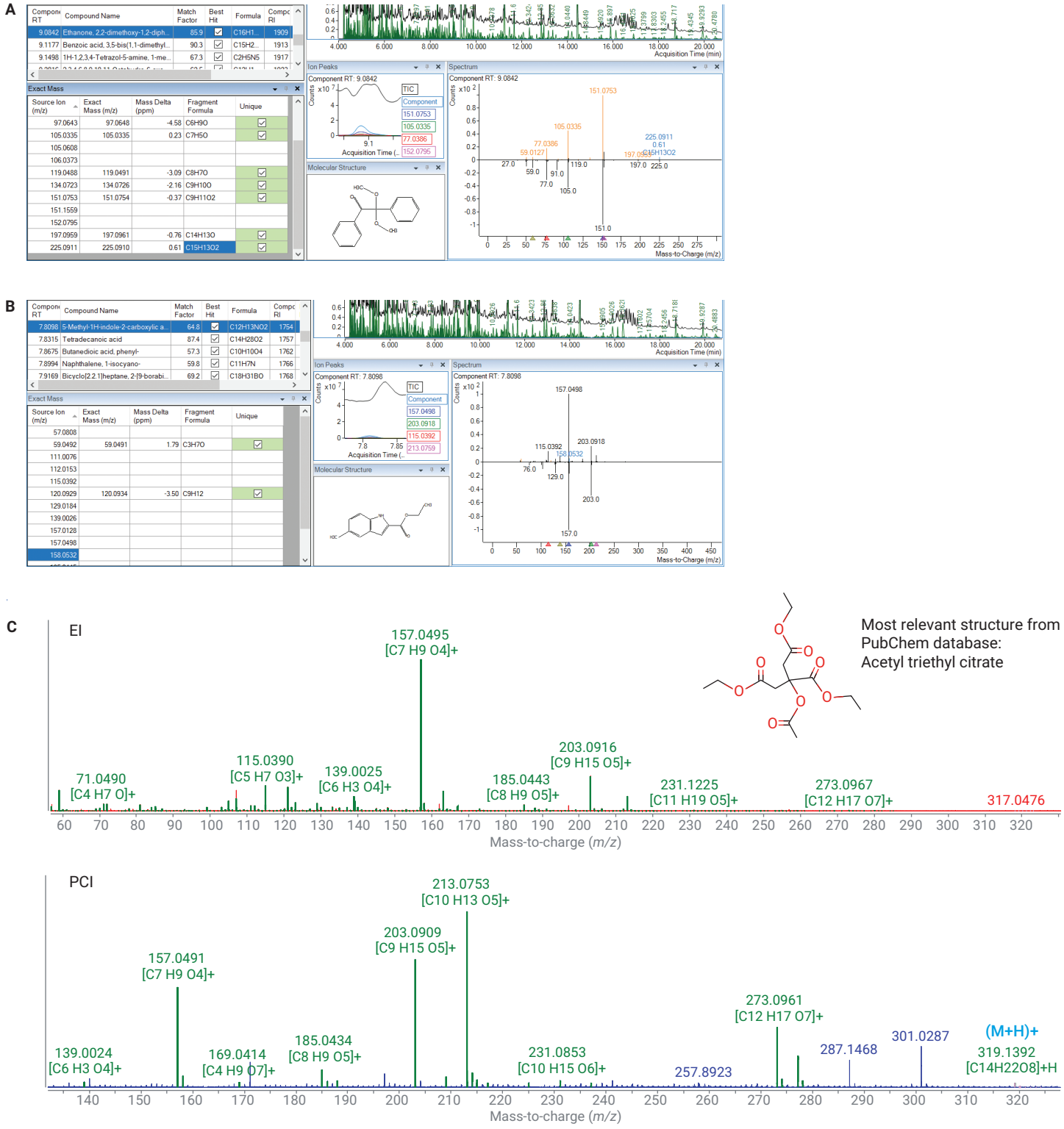


Figure 8. Compound verification and identification using Unknowns Analysis with the ExactMass feature: (A) confirmed ID, (B) rejected ID, and Fragment Formula Annotation in Agilent MassHunter Qualitative Analysis of EI (C, top) and PCI (C, bottom) spectra for the compound with the rejected ID.

Conclusion

An EI and NCI environmental contaminant suspect screening approach combined with nontargeted screening was demonstrated using the Agilent 7250 GC/Q-TOF system. A few compounds of interest, including pesticides such as flurprimidol, paclobutrazol, azoxystrobin, and chlorantraniliprole, were identified predominantly in the samples associated with some degree of toxicity.

The nontargeted approach helped to identify additional compounds that might be associated with the mortality of *C. dubia*. While the nontargeted approach is unlikely to detect minor differences in the levels of trace compounds, unlike the suspect screening approach, it is able to find potential contaminants outside of the accurate mass PCDL.

References

1. SANTE/11945/2015. Guidance document on analytical quality control and method validation procedures for pesticide residues analysis in food and feed (**2015**).
2. Food and Drug Administration. Department of Health and Human Services. Memorandum: Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data within the Office of Foods and Veterinary Medicine (**2015**).
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