Using zebrafish embryo bioassays combined with high-resolution mass spectrometry screening to assess ecotoxicological water bodies quality status: a case study in Panama rivers

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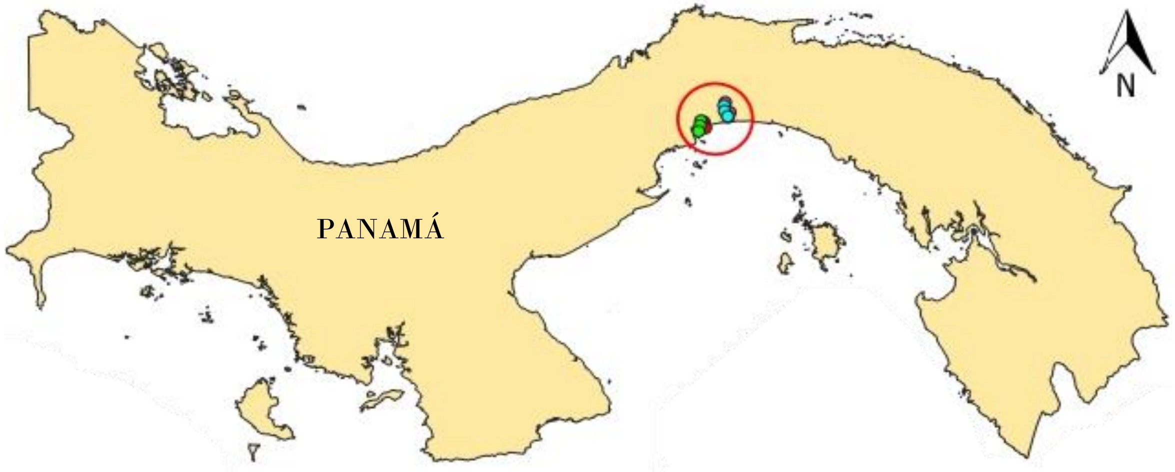
HIGHLIGHTS:

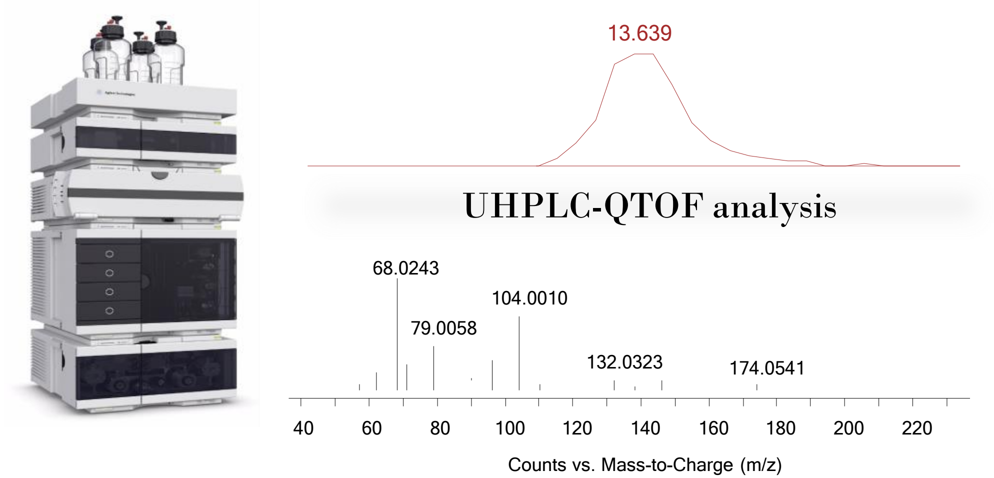
- 68 CECs were detected in the rivers of Panama considered
- A good correlation between anthropic sources and ecotoxicological and chemical data was observed
- Zebrafish embryo bioassays combined with LC-HRMS is a robust approach for the assessment of the ecotoxicological status of water bodies

Graphical Abstract

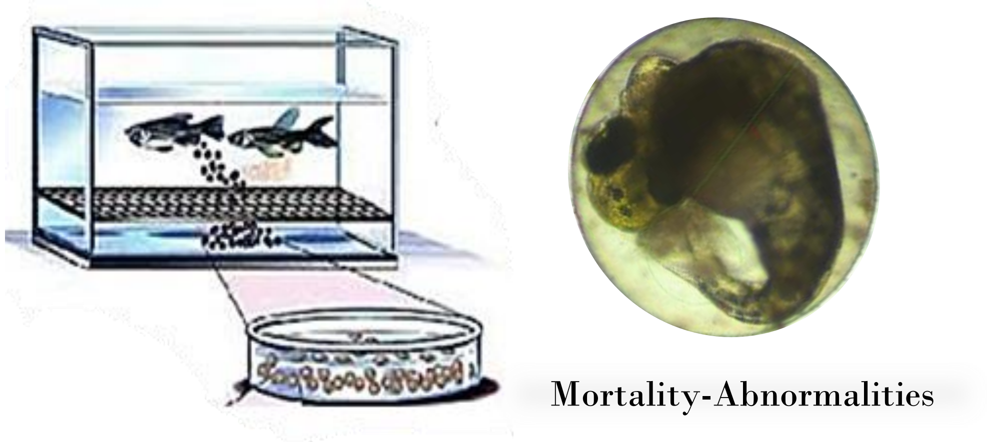
Water Quality of Panamá rivers



CECs - UHPLC-HRMS suspect screening



FET - Zebrafish embryo bioassays



Abstract

Several studies show that many water bodies in developing countries are increasingly affected by anthropogenic pressure, such as agricultural activities, domestic and industrial wastewater. However, data is scarce in several of such countries, including Panama. Thus, in this work, the ecotoxicological status of selected rivers in Panama with distinct input sources were evaluated using the zebrafish (*Danio rerio*) embryo bioassays combined with a liquid chromatography-high resolution mass spectrometry screening of contaminants of emerging concern (CECs), using a library of over 3200 chemicals. A total of 68 CECs, including pharmaceuticals and metabolites, pesticides and several industrial chemicals, could be tentatively identified. Additionally, the zebrafish embryo bioassays showed a significant increase ($p<0.05$) in embryo mortality/abnormalities when incubated with water samples from two rivers, Matasnillo and Curundú (47.5% and 32%, respectively). Importantly, a positive correlation between ecotoxicological endpoints and some of the detected CECs was observed. The findings demonstrate that both rivers are under strong anthropogenic pressure, and therefore, management actions are urgently needed to decrease their level of contamination. Overall, this study further supports the use of the zebrafish embryo bioassay as a fast, high throughput approach for screening the toxicity of water samples, and highlights the advantages of combining ecotoxicological assays with high-resolution mass spectrometry to an expedite assessment of the ecotoxicological status of water bodies.

Keywords: Emerging contaminants, Quadrupole-time-of flight mass spectrometry (QTOF), screening, risk assessment, toxicity testing, *Danio rerio*.

1. Introduction

Anthropogenic activities such as agricultural, industrial practices and domestic effluents play an important role in the contamination of water bodies (Castro et al., 2004; Solé et al., 2008). These practices generate pollution and have altered the water cycle in many rivers, causing a global concern linked to their potential impact on wildlife and human health (Capela *et al.,* 2016; Richardson and Ternes, 2018).

Water bodies receive discharges of wastewater effluents that lead to the introduction of trace levels of various organic pollutants such as pharmaceuticals, hormones, personal care products, pesticides and disinfection byproducts, and many other contaminants of emerging concern (CECs) which have now also become prominent agents of research interest for environmental scientists (Rodil *et al*., 2012; Santos et al., 2016; Wilkinson *et al*., 2017; Schulze *et al*., 2019). This is due in large part to the revolutionary development of resources and technologies that have produced more chemicals and compounds that can hold potential environmental risks (Castro and Santos, 2014; Richardson and Kimura, 2016;). On the other hand, wastewater treatment plants (WWTP) work well in removing certain classes of contaminants but can be ineffective in removing some of the aforementioned pollutants and, therefore, constitute important sources of contaminants in the aquatic environment (Rigobello *et al.*, 2013, Escada *et al.,* 2018, 2019).

In Panama, due to the lack of planning, budgeting, and use of irresponsible practices, some water reservoirs became seriously polluted and the available supplies of water, such as rivers, lakes, streams, and underground aquifers, continue to be contaminated often causing a shortage of drinking water (Barranco, 2013). Factors that generate water pollution include the discharge of industrial wastewater and domestic sewage, agrochemicals, inefficient application of the specific norms for residual water discharge, direct and indirect discharges of hydrocarbons by ships that use the Panama Canal, sedimentation accumulation, poor promotion of pollution prevention and lack of environmental education, and scarce data on water quality especially in rural areas (Vega, 2012).This is more evident in the Rivers of the metropolitan area such as Curundú, Matías Hernández, Juan Díaz or Matasnillo.

Due to the increasing detection of several pollutants in water bodies in recent decades, either due to increased use or advances in analytical methods, it is virtually impossible to measure all potential chemicals. In this context, liquid chromatography combined to high-resolution mass spectrometry (LC-HRMS) screening methods have already been shown to play a relevant role in detecting as much chemicals as possible with little or no aprioristic selection (Montes *et al*., 2018; Gago-Ferrero *et al.,* 2015; Hernández *et al*., 2012). Furthermore, an increasing number of studies has been focusing on the environmental fate and impact in non-target organisms (Brausch and Rand, 2011, Coimbra *et al.,* 2015, Neuparth *et al*., 2014, Wilkinson *et al*., 2017). CECs are not commonly monitored, particularly in developing countries, despite having the potential of entering the environment and causing adverse ecological and/or human health effects (Silva and Collins, 2011; Rodil *et al.,* 2012; Masiá *et al*., 2015; Wilkinson *et al*., 2017; Richardson and Ternes, 2018). Hence, the use of ecotoxicological assays as an additional tool in the determination of water quality has been implemented in several regions. The use of embryo bioassays as a prioritizing tool for screening the toxicity of water courses, enables the acquisition of relevant ecotoxicological information with limited resources in a short time-frame, replacing the use of adult/juvenile animals, providing ecologically relevant information (Capela et al., 2020a). Among the standardized embryo bioassays, a recent high-throughput approach involves the use of zebrafish (*Danio rerio*) embryos in hazard and risk assessment. Zebrafish displays many features essential for a model animal test such as the rapid external embryo development, larval transparency, high genetic and physiological homology to mammals and easy laboratory maintenance and manipulation (Scholz *et al.,* 2008; Sipes *et al*., 2011; Van den Bulck *et al*.,2011; Dai *et al.,* 2014; Torres *et al.,* 2016; Macedo *et al*., 2017; Capela *et al*., 2020a, b). Thus, combining both zebrafish embryo bioassays with LC-HRMS (suspect) screening is expected to provide a high-throughput approach for the ecotoxicological status of freshwater bodies, without aprioristic constrains, which is performed here as a proof of concept. Given the evidences of contamination of several water courses in Panama, rivers with distinct input sources from its metropolitan area were selected as a case study.

2. Materials and Methods

2.1 Site selection and sampling

The study area includes four river basins located on the Pacific watershed of Panama, with different input sources (Figure 1). The sampling points were taken as reference to the Environmental Impact Study category III of the Sanitation of the City and Bay of Panama (Ministerio de Salud-MINSA Panamá, 2009). For each river, three different sampling sites were selected: upper course (UC), middle course (MC) and lower course (LC). The Tocumen and Tapia rivers have been reported to support less input sources of effluents in comparison to the Matasnillo and Curundú rivers (Ministerio de Salud-MINSA Panamá, 2009). Water samples were collected in January 2019 (summer); the samples of the Matasnillo river were collected on 1/15/2019, the Curundú river on 1/17/2019, Tocumen and Tapia river on 1/18/2019. At each sampling site the coordinates were taken; the samples were collected in the water column (half a meter deep) and stored in pre-sterilized NalgeneTM bottles. Measurements of pH, dissolved oxygen and temperature were taken in the field with the multiparameter meter HANNA-H1 9828. Each sample was collected and stored into 500 mL bottles (for ecotoxicological assays at CIIMAR) and 250 mL (for chemical determinations at the University of Santiago Compostela). After collection, the samples were immediately refrigerated at 4 °C and preserved at -80 °C until analysis.

The Matasnillo river basin is located towards the center of Panama City, the Pacific slope, with a length of 7.45 km, with a latitude of 85°59'00"N79°31'00"W. Industries that possibly contribute to the degree of contamination of this river include poultry meat factories, dry food (flours, etc.), metal, and beer factories, among others. In addition, this river also receives domestic effluents from the large urbanizations in the vicinity (MINSA,2009). The Curundú river has 10.74 km of length, 85°58'00"N79°33'00"W. The quality is affected by the intense entry of domestic and industrial discharges. The Tocumen river comprise 20.9 km of length; 9°03´9°10´N79°22¨ 79°23´W. This river crosses lands of little human occupation at present until arriving at the urbanized area of Tocumen. It then runs parallel to the Tocumen International Airport, crossing crop fields, to flow into the mangrove area in Panama Bay. The Tapia river has 17.2 km of length, 9°03´9°08´N79°27¨W. This basin has an elongated shape, following Southwest direction, and then pouring its waters into the Tocumen river, which flows into the Bay of Panama. The middle part presents some discharges of industrial effluents of factories of oils, glass, dry food, ice cream. In its lower part, it receives some domestic effluent discharges from the urbanization near the river.

2.2 Zebrafish embryo bioassays

This work was carried out using the standardized zebrafish Embryo Acute Toxicity (FET) Test- 236 of The Organization for Economic Co-operation and Development (OECD) with modifications according to Espíndola *et al.,* (2019).

Newly fertilized zebrafish eggs were placed in 24-well plates (1 embryo per well) and exposed to the water samples and controls for a period of 96-120 hours (hpf). The 24-wells plates were incubated at 26±1 °C during 120 hours under the same photoperiod conditions as the zebrafish stock. A magnifying glass with (LEICA E24) was used for observation and 1 fertilized egg (1-2 hpf) was placed in each well previously filled with 2 mL of river water as presented in Figure S1, following the OECD 236 protocol. For each river water sample, two independent replicate plates with five replicates were set. Each replicate consists of four embryos. A total of 40 embryos per condition were analyzed. Water was changed daily. Every 24 hours up to four apical observations were recorded as indicators of lethality and sublethality, coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tailbud from the yolk sac and others. In addition to the apical endpoints, development abnormalities were also recorded according to Torres *et al*., (2016).

Mortality was assessed by daily recordings during the entire exposure period and coagulated eggs or death embryos were removed. The effects of the exposure were evaluated at four time-points 48, 72, 96 and 120 h hpf. The observation periods were selected based on a set of characteristics present in embryos at these stages of development (Kimmel *et al*., 1995). The embryo development and abnormalities were observed using an inverted microscope (Nikon Eclipse TS100) equipped with a digital camera (Nikon D5-Fi2) and a microscope camera controller (Nikon's Digital Sight DS-U3). Morphological abnormalities were rated as abnormalities in head, eyes, tail, or yolk-sac, developmental delay, abnormal cells, pericardial edema, opaque chorion, excess or lack of pigmentation, lateral position, reduced mobility, and involuntary movements; then, the total abnormalities were expressed as the percentage of embryos with one or more abnormalities in comparison to the control. (Lammer *et al*., 2009; OECD, 2013; Torres *et al.,* 2016).

2.3 Physico-chemical parameters and nutrients

The parameters temperature, oxygen and pH were measured in situ with a Hanna HI 9828 (Woonsocket, RI, USA) multiparameter portable meter. A 30 mL aliquot was used to photometrically (Palintest Photometer 7500, Gateshead, UK) measure ammonium, phosphate, nitrate and nitrite, with the Palintest Photometer 7500, according to the supplier instructions.

2.4 Chemical analysis

2.4.1 Sample preparation

Water samples were shipped frozen to Santiago de Compostela, where they were subjected to solid-phase extraction (SPE) within 48. Samples (200 mL) were filtered, spiked with 18 different isotopically labelled chemicals, used as internal standards (IS) and subject to SPE on Oasis HLB 200 mg (Waters, Milford, MA, USA) cartridges. These were eluted with methanol, which was evaporated and made to a final volume of 0.5 mL. These samples were then analyzed on an LC-quadrupole-time-of-flight-HRMS Agilent system in both data-dependent and data-independent acquisition modes (DDA and DIA). Chemicals which were tentatively identified were integrated and normalized to an IS, in order to account for matrix effects. Procedural blanks were run with the samples and chemicals detected in the blanks were excluded.

Further details on sample preparation and analysis are provided in the Supporting Information (Text S1).

2.5 Statistical analysis

Data were analyzed using SPSS version 21.0 software. All data were tested for homogeneity and normality using Levene’s and Kolmogorov-Smirnov test. For the zebrafish bioassay, given that these assumptions were not met, differences between treatments were tested for significance by means of non-parametric Kruskal-Wallis test followed by Binomial test for multiple comparison adjusted with Bonferroni correction between groups that compare the control groups and each of the treatment groups (Bellas *et al*., 2005). The adjusted-p-values were considered for multiple comparisons. To fulfill assumption of independence of data, the results were analyzed at 96 hpf.

Principal components analysis (PCA) was performed using The Unscrambler version 11.0 (CAMO Software AS, Oslo, Norway) by using the chemical normalized response of the analytes that could be detected in all samples together with the 96-hpf mortality and the total abnormalities ecotoxicological endpoints. Variables were centered and autoscaled in all cases.

3. Results and Discussion

3.1 Physico-chemical parameters and nutrients

Since no parametric values for physico-chemical parameters and nutrients are available in the Panama regulation, we used here for a comparison purpose the criteria established in the European Water Framework Directive (WFD, 2000) for a good ecological status (Martinez-Haro *et al.,* 2015). Given the differences in the rivers between Panama and Europe, caution should be taken in the interpretation of the results. All rivers analyzed in this study, show the nitrates and nitrites values within acceptable concentration considering the standards of the WFD in accordance with the classification criteria for the establishment of the good ecological status in rivers Directive 2000/60/EC (Table 1). In contrast, phosphates exceeded the WFD parametric values in all samples, being particularly high in MC and LC. Similarly, ammonia was also above the WFD threshold in MC and LC samples in the four rivers, being particularly high in the Matasnillo and Curundu rivers, which are those receiving a higher anthropic pressure. High levels of ammonia have been reported to be toxic to fish, and an increase in the rate of zebrafish embryo abnormalities was observed when incubated with surface water with high ammonia loads (Chen et al., 2015). Additionally, the oxygen concentration, showed similar values between the middle and lower course. All rivers presented temperature and pH values within the established range according to its hydrogeographic components of the region.

3.2 Contaminants of emerging concern

Sixty-eight different compounds were tentatively identified (identification level 2a, according to Shymanski *et al.,* 2014) in total, as summarized in Table 2, many of which are related to urban wastewater discharges, but also to agricultural practices to a minor extent. Of the detected CECs, 43% are pharmaceutical products, 16% pesticides, 10% metabolites of pharmaceuticals or drugs of abuse, 9% plasticizers and 22% correspond to other diverse groups of chemicals. Furthermore, 25 compounds could be detected in all samples (Table 2), among which pharmaceuticals, pesticides and plasticizers represented ca. 24% each, 16% metabolites and 12% the remaining groups. Additionally, 10 compounds (amantadine, atenolol, betaxolol, dexpanthenol, gabapentin, irbesartan, salicylic acid, sucralose, thymotic acid and triamterene) were detected in 11 out of the 12 samples. A recent review (Peña-Guzmán *et al.,* 2019) highlighted the limited number of studies on CECs in Latin America, where also most of the research was performed on Mexico, Brazil and Colombia, while in Central America, besides Mexico, some research was performed in Costa Rica and Guatemala. Furthermore, those publications have been performed with targeted methods, so that many of the here reported chemicals were not investigated.

Considering sample location, no clear differences can be depicted from the number of the detections, since the detection rate ranged from 58 in Matasnillo river to 66 compounds in Tapia river (Figure 2a).

As explained in 2.4 and Text S1, the areas of the chemicals detected were then integrated and normalized by the area of an IS, as compiled in Table S2. This relative signal does not account for concentrations, but provides normalized data that corrects for variability among different sample matrixes due to the well-known susceptibility of LC-MS to matrix effects (Reemtsma and Quintana, 2006). However, it can be used as a proxy of particular interest when looking at the differences between samples for the same chemical. Figure 2b, where the sum of such (log-scaled) normalized areas is displayed. Although there is a large uncertainty here due to the different responses of different chemicals and SPE extraction efficiency (Montes et *al.*, 2017), still higher response is normally obtained for several chemicals (see Table S3), and hence it can provide a qualitative idea of which samples have higher cumulative concentrations of CECs. Furthermore, for those seven chemicals whose labelled IS were available, estimated concentrations (ng L^{-1}) were calculated from a calibration curve and presented in Table S3. It must be noted that those concentrations are only an approximation, since the IS is expected to correct for SPE and instrumental variations, but a validation of the method would be required. As it can be observed, the MC of the Matasnillo river would correspond to the most polluted sample in terms of estimated CECs concentrations. This agrees with the mortality observed in the zebrafish bioassay (see Figure 3). Moreover, several of the compounds found in that sample showed the higher values of predicted toxicity for fishes (see Table 2), such as fipronil (EC_{50}: 1.96E-08 mol L^{-1}), tetramethrin (EC_{50}: 2.20E-07 mol L^{-1}) and 8-hydroxyefavirenz (EC_{50}: 2.75E-07 mol L^{-1}). The Curundú river is then the second river with higher cumulative estimated concentration of organic micropollutants, the signal of the compounds found in the UC being slightly higher than the LC. Yet, there is a significant difference between the toxicities of detected compounds in the UC and LC of the Curundú river. Actually, some compounds with high toxicity values (e.g. mycophenolic acid (EC_{50}: 7.01E-07 mol L^{-1}), losartan (EC_{50}: 5.53E-07 mol L^{-1}) or ibersartan (EC_{50}: 7.35E-07 mol L^{-1}) were not found in the UC, while fipronil (the compound predicted to be more toxic) was detected with higher normalized signals in the LC (Table S2). In general, lower cumulative normalized signals were obtained along the courses of Tocumen and Tapia rivers.

3.3 Zebrafish embryo bioassay

The water samples from the middle course of the Matasnillo river lead to a significant ($p<0.05$) increase in the mortality rate of zebrafish embryos, i.e.,47.5% at 96 hpf. in comparison with the control treatment (Figure 3a).

In addition to the lethal effects observed, sublethal effects were also recorded in the embryonic development. Figure S2 displays different abnormalities observed in embryos tested with samples from all rivers. In the Matasnillo river, 24% of total abnormalities was observed, i.e, mostly scoliosis (6.5%) and yolk-sac enlarged/malformed (6.5%), particularly in the middle and low course. The abnormality rates were significantly elevated for the lower course of the river in comparison to control and to the upper course (p<0.05) (see Figure 3b).

In the Curundú river, a significant increase (p<0.05) in the mortality rate at 96 hpf was observed between the lower course of the river (32.5% mortality) and control.; In contrast, 5% mortality was recorded in the middle course at 96 hpf and 10% in the upper course. Morphological abnormalities were also observed in the embryos exposed to the water from the Curundú river, i.e, 38% for the lower course, including abnormalities such as pericardial edema (the most frequent) in the lower course (12.5%) and yolk-sac enlarged/deformed (4%) Fig.3d. Total abnormalities in *D.rerio* embryos exposed to water samples of Curundú river differed significantly (p<0.01) between groups at 96 hpf: LC-UC and LC -control.

In the Tocumen river the highest mortality rate observed at 96 hpf was 17.5% in embryos incubated with water samples from the lower course of the river. This mortality rate did not differ significantly from the control treatment (p=0.249) (see Figure 3e). Morphological abnormalities were also observed in the embryos exposed to waters of this river (Figure 3f), which present a 20% of total abnormalities in the middle course, i.e., yolk-sac enlarged/malformed with 6% (the most frequent abnormality); followed by head deformed with 4%; this river also presented abnormalities of tail deformed with 4% and 2% in the middle and lower course, respectively. However total abnormalities in *D. rerio* embryos exposed to water samples of Tocumen river did not differed significantly from control (p>0.362).

Embryos incubated with water samples from the Tapia river showed low rates of mortality (<10%) at all locations and time-point observations (see Figure 3g). Although the lethal effects were lower compared to the other analyzed rivers, there were also cases of abnormalities with a 11% in embryos exposed to water samples of the Tapia river from the MC. The mortality rate and total abnormalities did not differ significantly from the control group (p=0.539 and p=0.562, respectively).

3.4 Integrated analysis of ecotoxicological and chemical data

To evaluate the differences between the rivers and courses, a principal component analysis (PCA) was applied with the normalized areas of those chemicals detected in all samples as well as 96-hpf mortality and total abnormalities as described in 2.5, Principal Components 1 (PC-1) and 2 (PC-2) explain a 61% of the total variance, as presented in the scores plot (Figure 4).

These scores plot shows also that there are no differences between the UC, MC and LC of the Tocumen and Tapia rivers. In contrast, the Matasnillo and Curundú samples clearly differ. In fact, the sample with lower values of PC-1 is the MC Matasnillo sample, followed by the LC Curundú. Again, these two samples correspond to those showing the higher mortality in the *D. rerio* bioassay and among those with higher cumulative concentrations, particularly of those chemicals expected to be more toxic. In fact, mortality is one of the variables with higher negative PC-1 loading, together with some pesticides such as terbuthazine, fipronil, terbutryn or DEET and other compounds such as paraxanthine (a metabolite of caffeine and tracer of sewage anthropogenic pollution) among others (Table S4). A Spearman's rank correlation analysis was also performed between the chemicals detected in all samples and 96-hpf mortality and total abnormalities. Significant correlations (p-value<0.05) were found between mortality and the pesticides carbendazim and terbuthylazine (p-values: 0.0191 and 0.0110, r_s: 0.7068 and 0.7668, respectively); and between total abnormalities and benzoylecgonine (the main metabolite of cocaine), paraxanthine (metabolite of caffeine) and umbelliferone (p-values: 0.0355, 0.0262 and 0.0201, r_s: 0.6338, 0.6702 and 0.7010, respectively). Figure S3 shows the scatter plots for those variables. All these chemicals do also have negative loading values in PC-1 (Table S4), as mortality and abnormalities. *In vivo* assays with zebrafish embryos exposed to some of the aforementioned chemicals, such as fipronil, tetramethrin, mycophenolic, terbuthylazine, carbendazim, benzoylecgonine, reported a negative impact in the development and/or changes at biochemical and behavior level (Plhalova et al., 2012; Chen et al., 2015; Andrade et al., 2016; Jiang at al., 2016; Mendis et al., 2018; Parolini et al., 2018; Park et al., 2020).

4. Final considerations

In the present work, four rivers from the Panama province, with different levels of anthropic pressure, were selected as a case study. The results obtained here, using a combination of high throughput embryo bioassays with *D. rerio* and a LC-HRMS screening with a library of over 3200 chemicals, confirms that the water samples from Matasnillo and Curundú rivers, located in the close vicinity of dense urbanization and industrial areas, show a high degree of toxicity and chemical contamination in contrast with the Tocumen and Tapia rivers. Further, the experimental approach was sensitive to discriminate the courses of the rivers under stronger anthropic pressure. Despite the screening of over 3200 chemicals, we cannot exclude the possibility that chemicals not included in the library could also contribute to the ecotoxicological outcomes. However, considering the main aim of the framework explored here, this is not an important shortcoming, as the aim of this work was to demonstrate the potential of the combined high-throughput approach to quickly identify water courses that could be under high chemical stress. Those locations identified in the screening could then go through a more detailed investigation to establish the sources of the stressors and implement mitigation and management actions.

Overall, this study further supports the use of the zebrafish embryo bioassay as a fast, high throughput approach for screening the toxicity of water samples, and highlights the advantages of combining ecotoxicological assays with high-resolution mass spectrometry to an expedite assessment of the ecotoxicological status of water bodies.

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CRediT authorship contribution statement

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Verónica Castro: Investigation, Methodology, Formal analysis, Writing - Review & Editing, Visualization

Raquel Chaves: Methodology, Resources, Writing - Review & Editing, Visualization

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Rosario Rodil: Methodology, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

José Benito Quintana: Conceptualization, Methodology, Formal analysis, Resources, Writing - Review & Editing, Supervision, Visualization, Funding acquisition

Maria da Natividade Vieira: Conceptualization, Methodology, Formal analysis, Writing - Review & Editing, Supervision, Visualization

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Supporting information: SI-1 (Supporting Information texts and Figures S1, S2 and S3), SI-2 (Excel, Tables S1, S2, S3 and S4)

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure Captions

Figure 1. Sampling sites in Panama rivers analyzed in this study; R1-Matasnillo river; R2-Curundu river; R3-Tocumen river; R4-Tapia river. (1: high course, 2: middle course, 3: lower course).

Figure 2. a) Number of CECs detected in the different courses of the four rivers.; b) Cumulative normalized response (sum of IS-normalized responses) on the different rivers and courses. N.B.: logarithmic scale.

Figure 3. Summary of zebrafish embryo bioassay results: **3a., 3c., 3e., 3g** - Mortality rate at the Matasnillo river-R1, Curundú river-R2, Tocumen river-R3 and Tapia river-R4, respectively, using the *D. rerio* embryo bioassay. **Fig.3b., 3d., 3f., 3h**. - Total abnormalities at the Matasnillo river-R1, Curundú river-R2, Tocumen river-R3 and Tapia river-R4, respectively. yolk enlarged/deformed (1), Yolk edema (2), Pericardial edema (3), Scoliosis (4), Non-development delay (5), Deformed tail (6), Totals abnormalities (7). Data are expressed as mean ± SD (n=10). Non-parametric ANOVA Kruskall-Wallis, followed by Binonimal test for multiple comparisons, adjusted with Bonferroni correction, performed on data at 96 hpf. (*)(**) indicates significant different from control at ($p<0.05$) and ($p<0.01$), respectively. UC-upper course (green bar), MP-middle course (brown bar), LC-lower course (orange bar), Control (gray bar).

Figure 4 – Principal components analysis scores plot for the two first principal components. LC: low course; MC: middle course; HC: upper course.

Figure 1

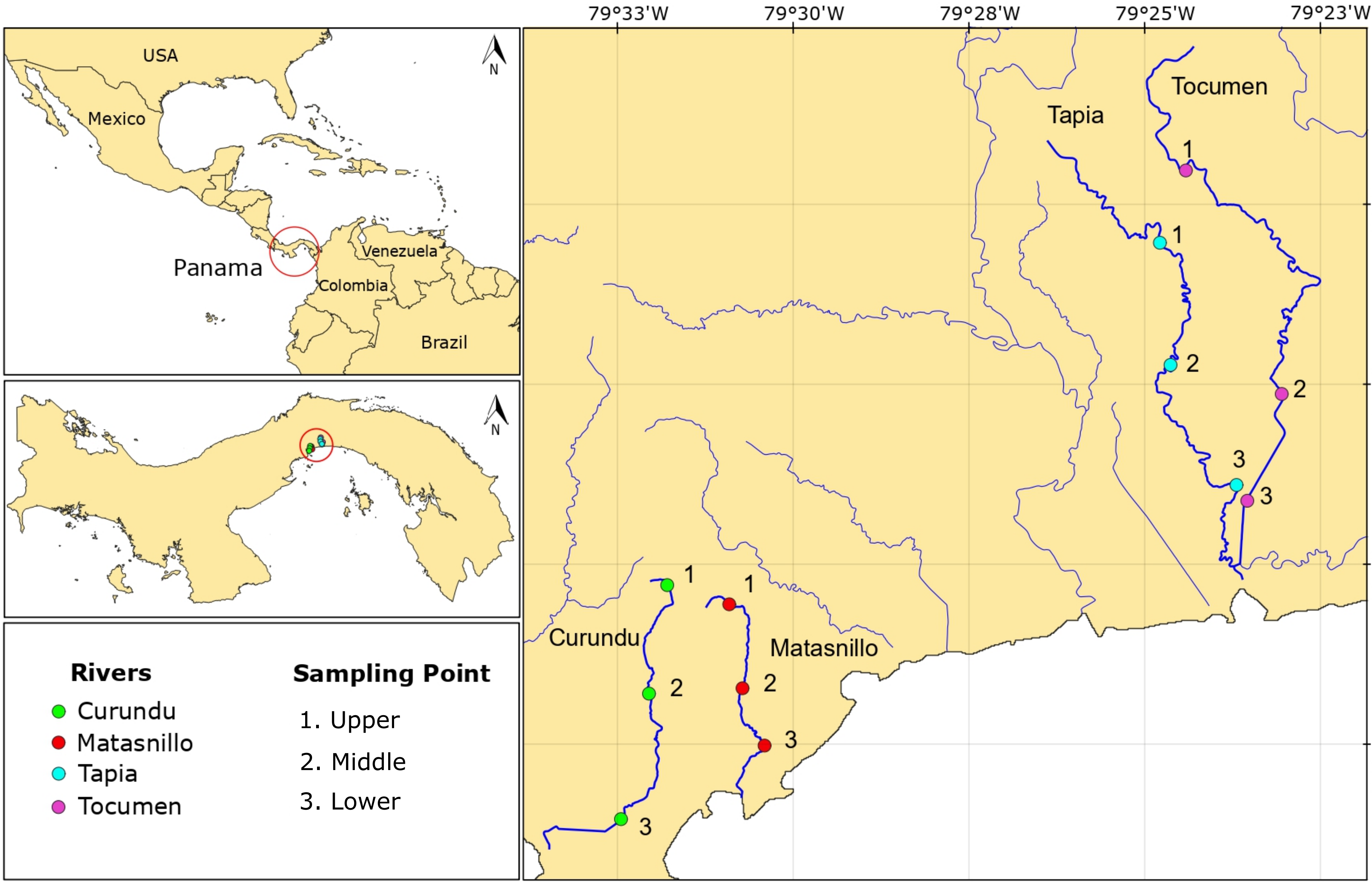
79°33'W
79°30'W
79°28'W
79°25'W
79°23'W
9°7'N
9°4'N
9°2'N
8°59'N
USA
Mexico
Panama
Venezuela
Colombia
Brazil
N
N
Tocumen
Tapia
Curundu
Matasnillo
1
2
3
Rivers
● Curundu
● Matasnillo
● Tapia
● Tocumen
Sampling Point
1. Upper
2. Middle
3. Lower

Figure 2

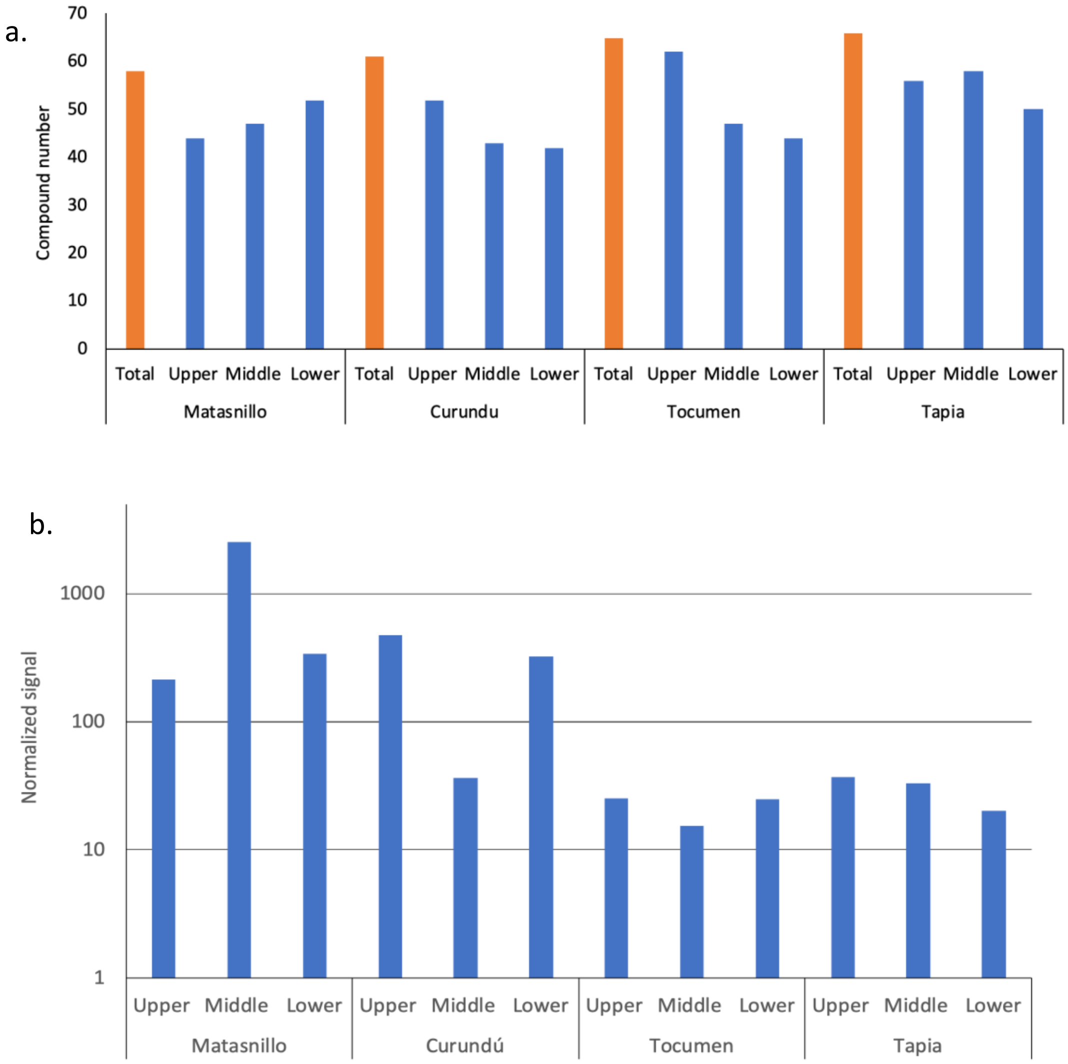
a.
Compound number
70
60
50
40
30
20
10
0
Total
Upper
Middle
Lower
Matasnillo
Total
Upper
Middle
Lower
Curundu
Total
Upper
Middle
Lower
Tocumen
Total
Upper
Middle
Lower
Tapia
b.
Normalized signal
1000
100
10
1
Upper
Middle
Lower
Matasnillo
Upper
Middle
Lower
Curundú
Upper
Middle
Lower
Tocumen
Upper
Middle
Lower
Tapia

Figure 3a

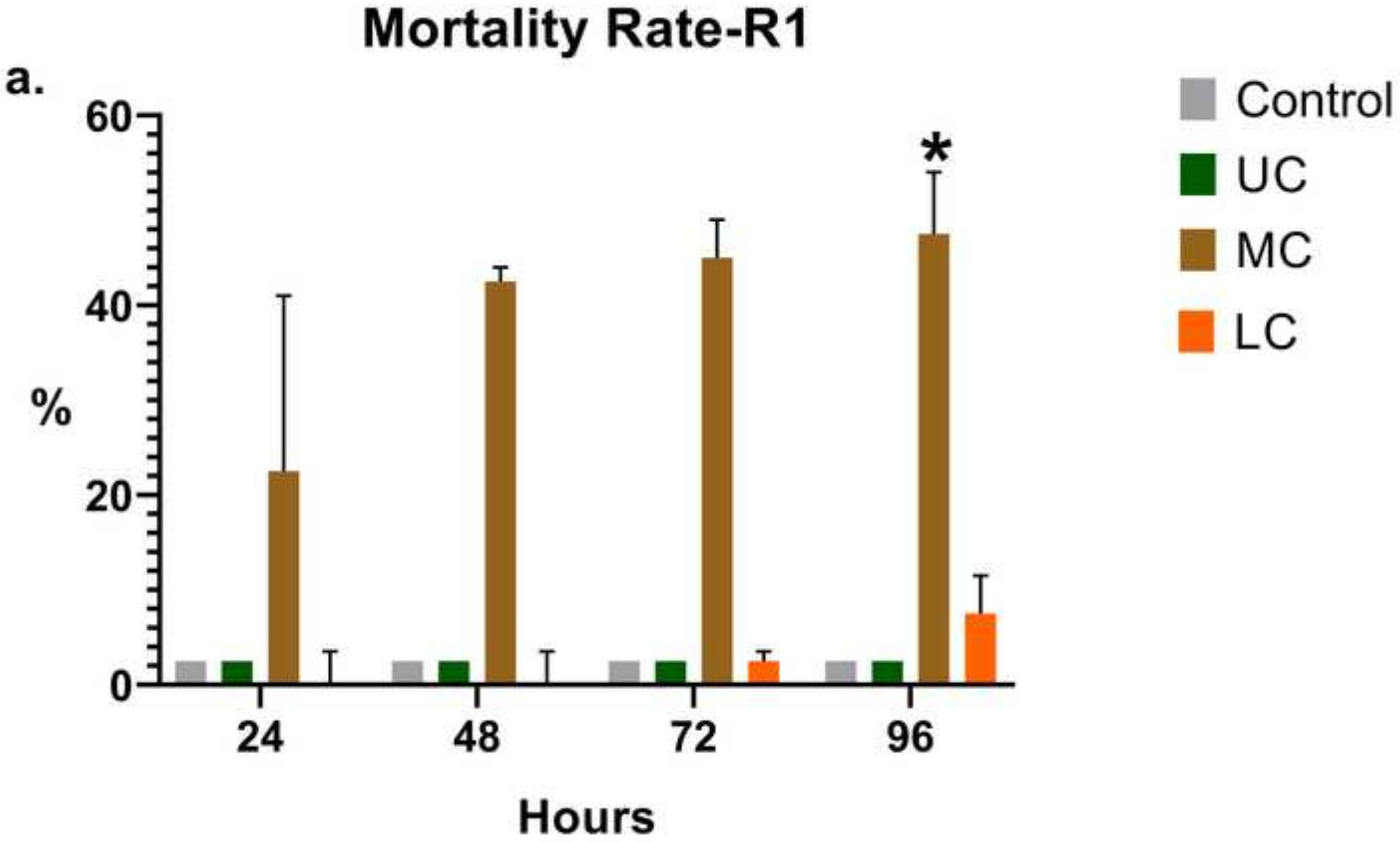

Figure 3b

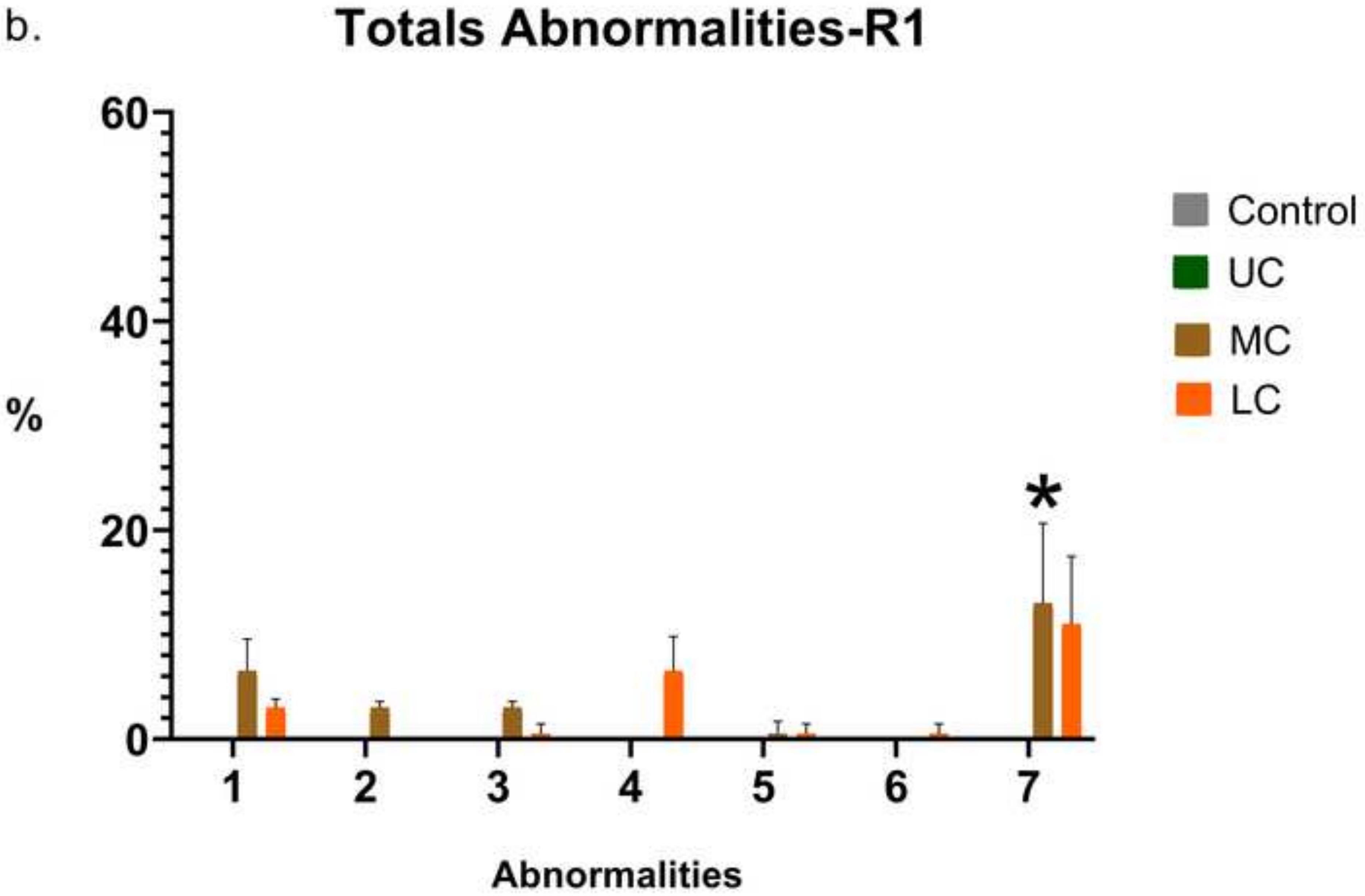
b.
Totals Abnormalities-R1
60
40
20
0
%
1
2
3
4
5
6
7
Abnormalities
*
Control
UC
MC
LC

Figure 3c

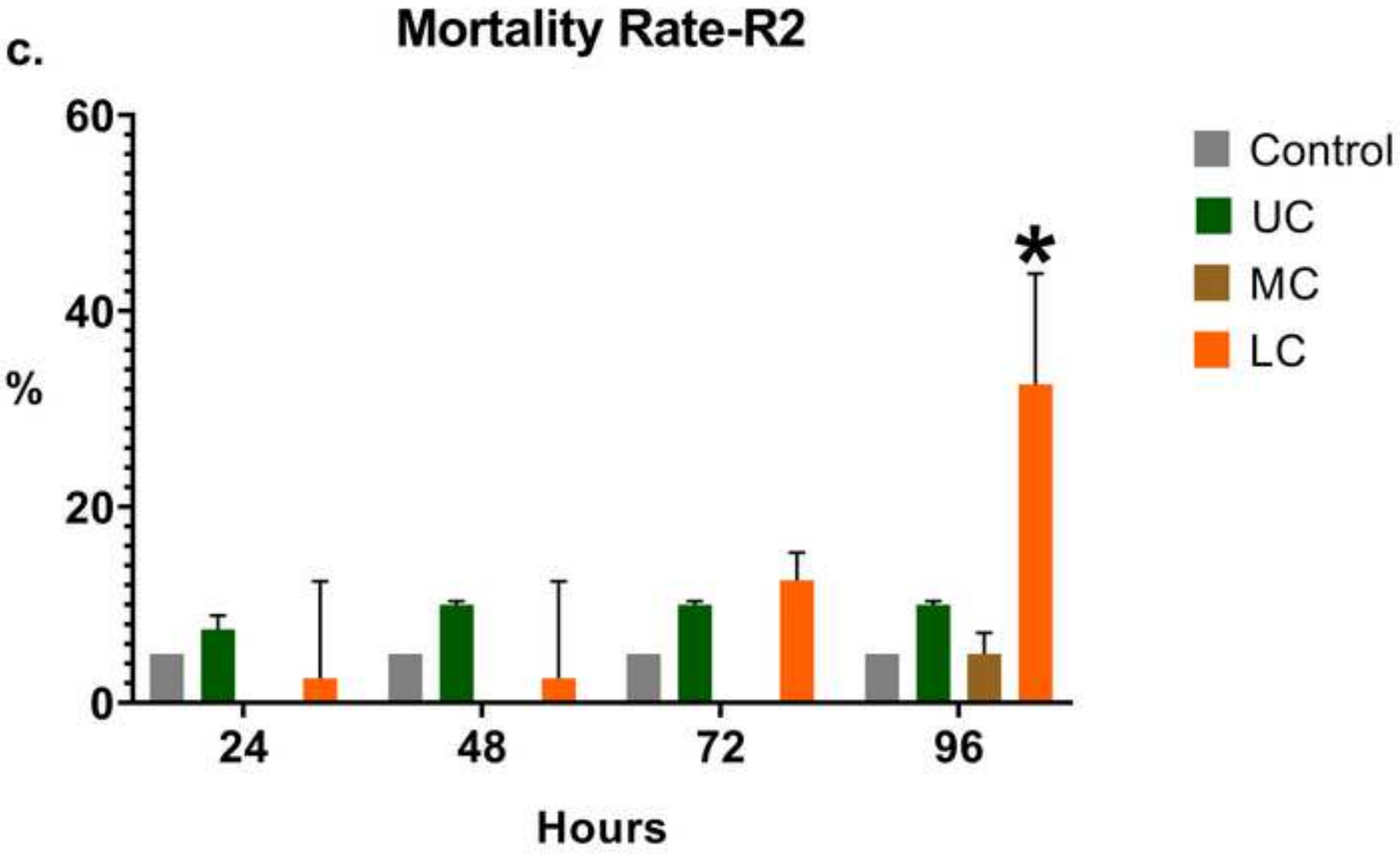

Figure 3d

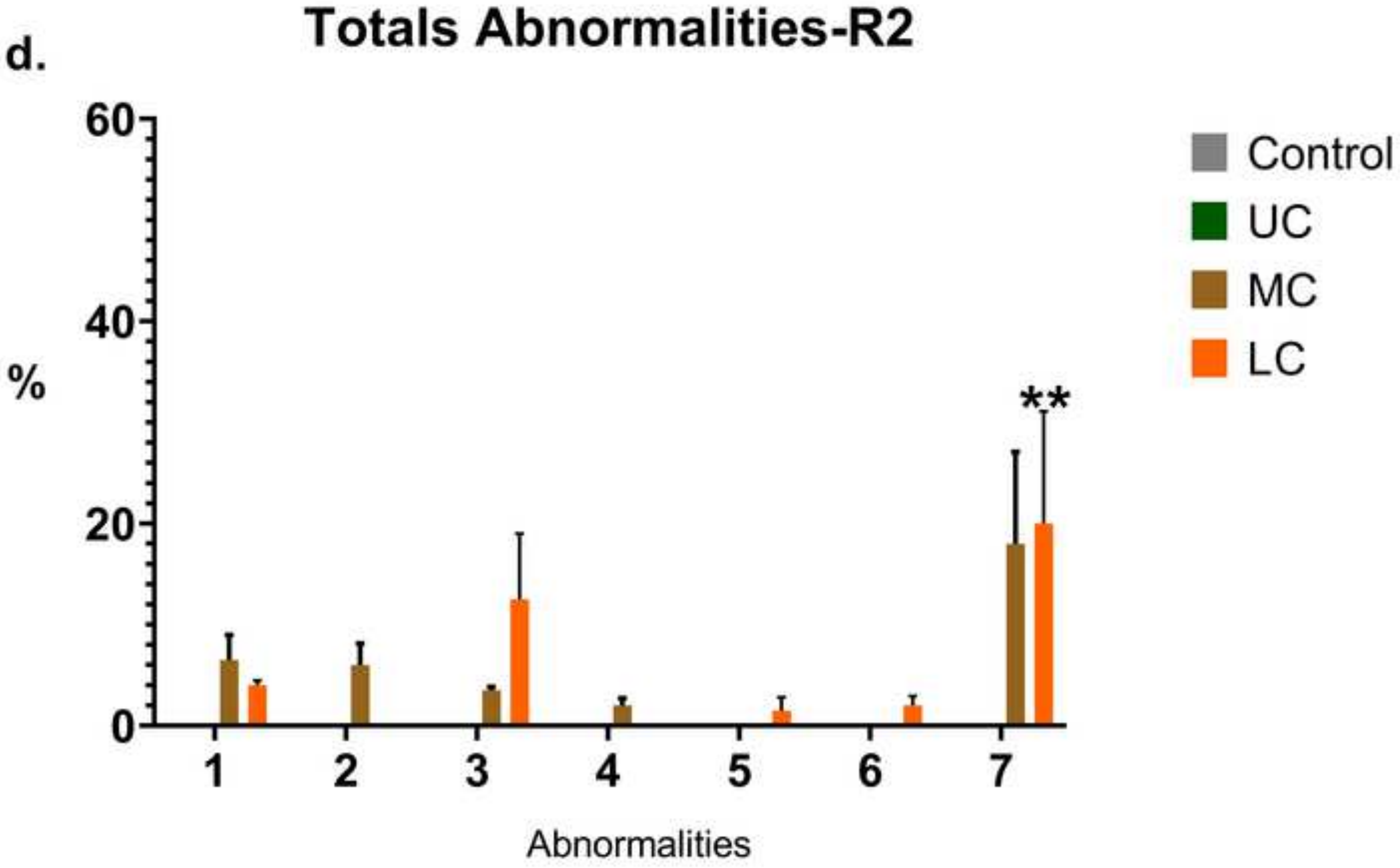
d.
Totals Abnormalities-R2
60
40
20
0
%
**
1
2
3
4
5
6
7
Abnormalities
Control
UC
MC
LC

Figure 3e

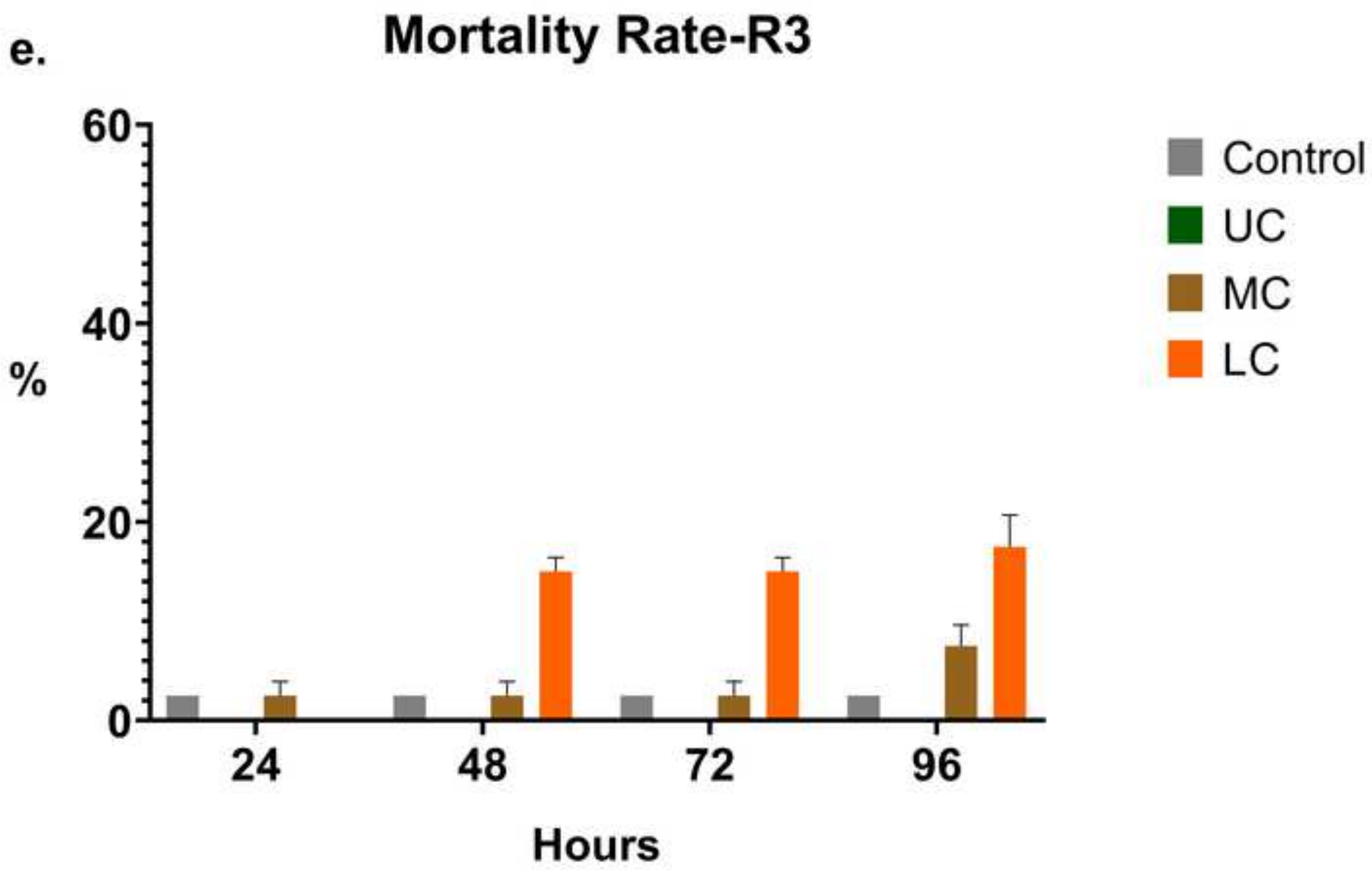

Figure 3f

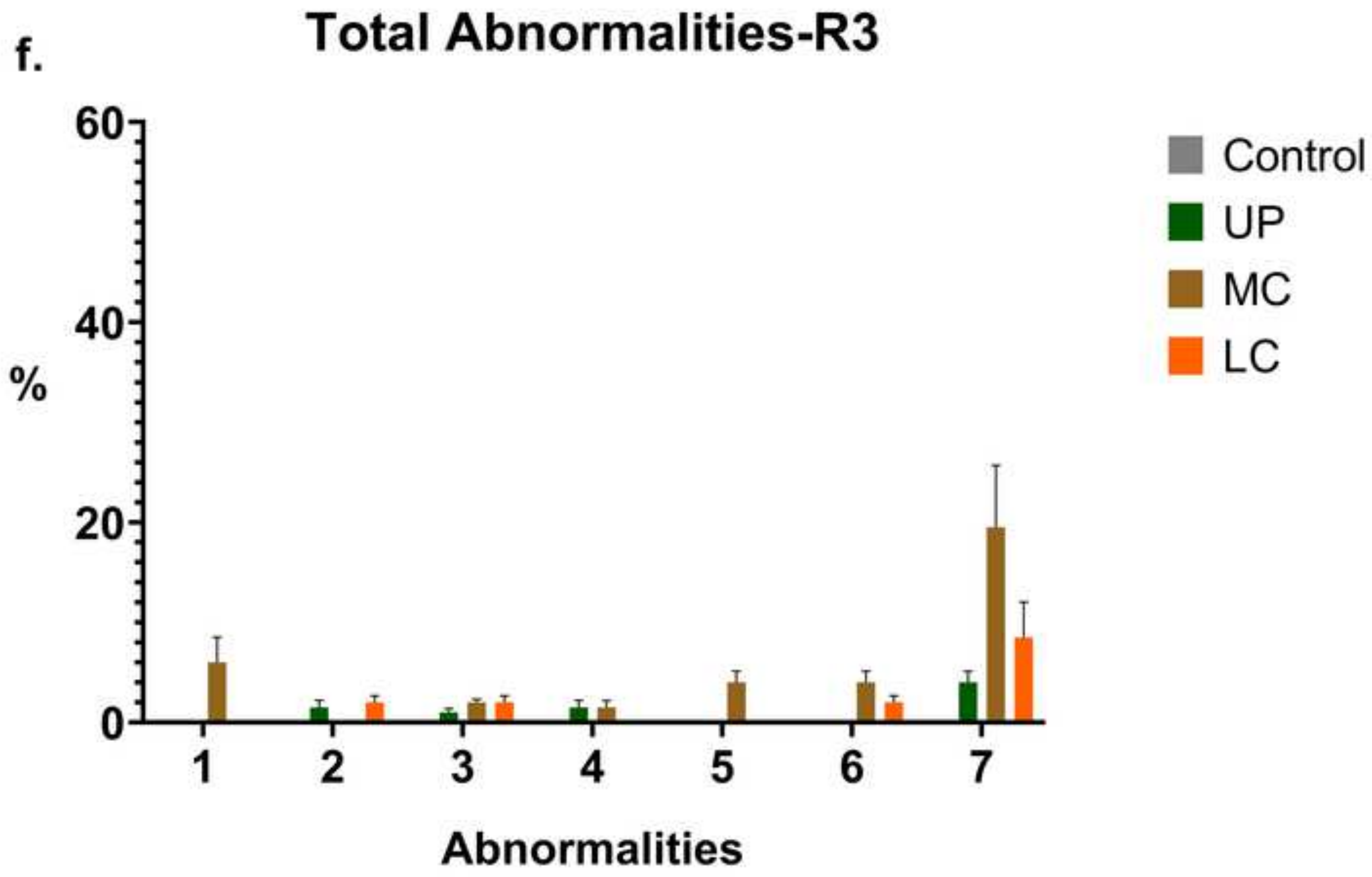
f.
Total Abnormalities-R3
60
40
20
0
%
1
2
3
4
5
6
7
Abnormalities
Control
UP
MC
LC

Figure 3g

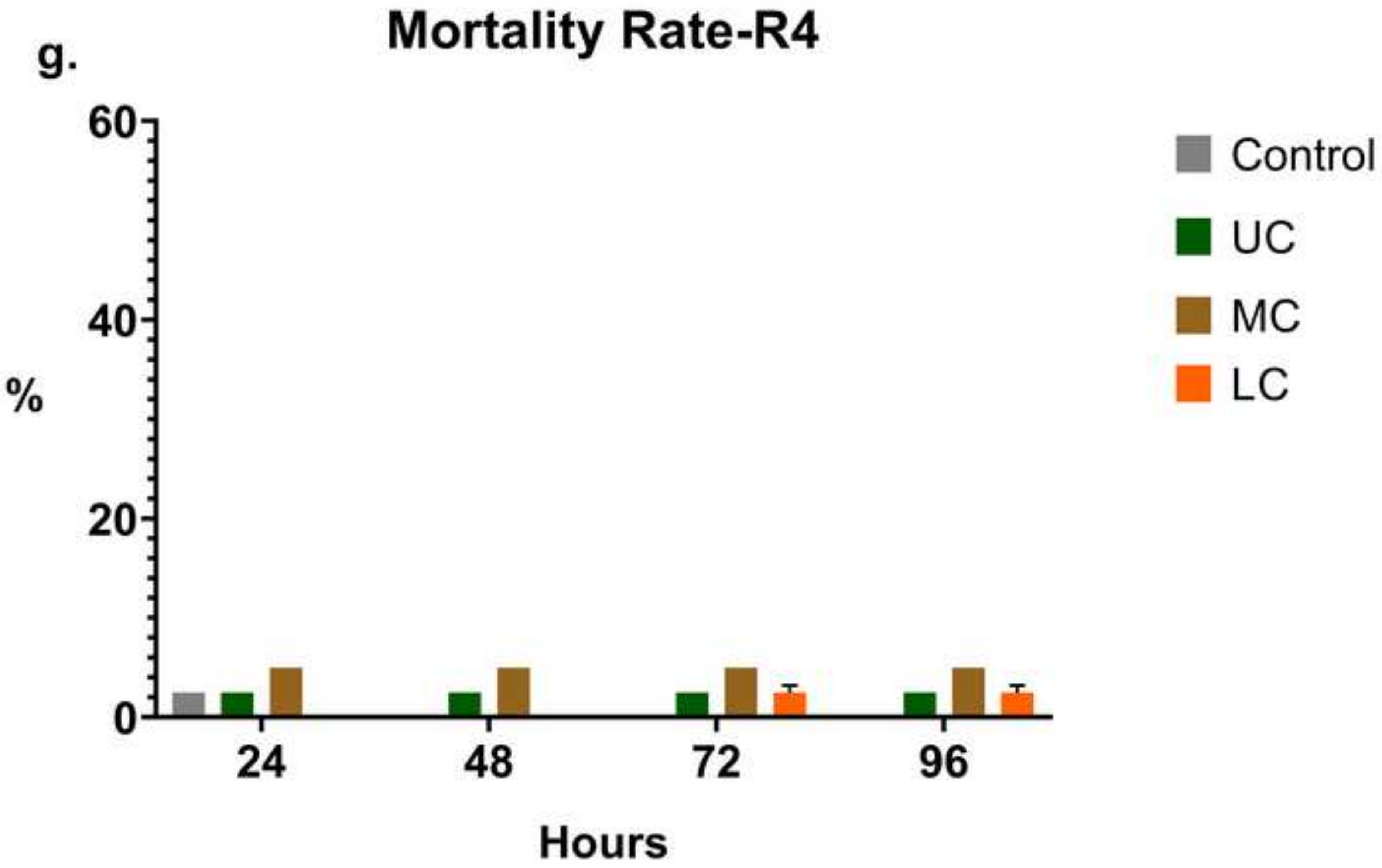

Figure 3h

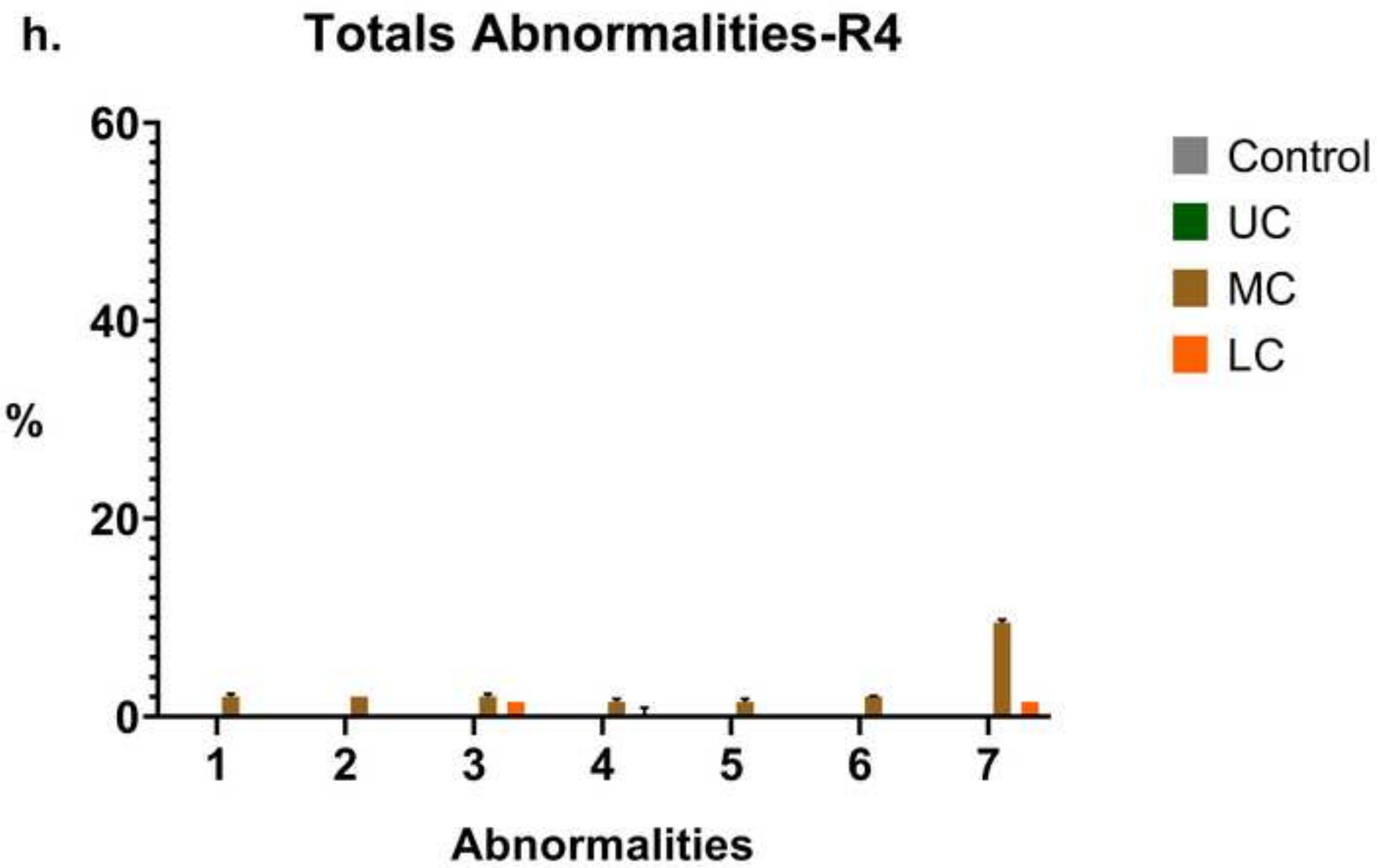
h.
Totals Abnormalities-R4
%
60
40
20
0
1
2
3
4
5
6
7
Abnormalities
Control
UC
MC
LC

Figure 4

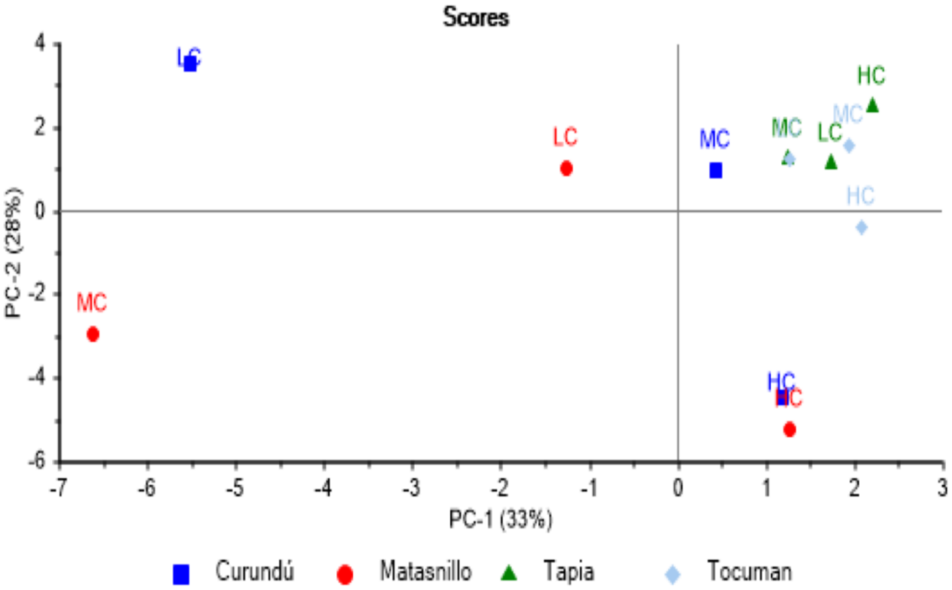
Scores
PC-2 (28%)
PC-1 (33%)
4
2
0
-2
-4
-6
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
LC
LC
MC
MC
HC
MC
LC
MC
HC
HC
HC
■ Curundú
● Matasnillo
▲ Tapia
◆ Tocuman

Table 1. Measured parameters of the studied rivers. UC: upper course; MC: middle course; LC: lower course. Bold: values above the WFD allowed limit.

Parameters	**Matasnillo river**			**Curundu River**			**Tocumen River**			**Tapia River**		
	UC	**MC**	**LC**	**UC**	**MC**	**LC**	**UC**	**MC**	**LC**	**UC**	**MC**	**LC**
NO_2^- (mg/L)	0.03	0.017	0.59	0.02	0.3	0.05	0.01	0.2	0.14	0.04	0.13	0.07
NO_3^- (mg/L)	4.3	0.195	2.8	1.32	1.42	4.4	3.4	2.3	2.04	3.4	2.5	1.98
PO_4^{3-} (mg/L)	**0.43**	**2.75**	**2.75**	**0.83**	**2.32**	**2.75**	**0.26**	**1.31**	**1.8**	**1.01**	**1.31**	**2.75**
NH_3 (mg/L)	0	**11**	**11**	0.92	**4.62**	**11**	0.63	**1.85**	**2.92**	0.91	**2.61**	**4.41**
pH	7.41	6.74	6.96	8.72	8.89	8.1	7.51	7.65	7.79	7.64	7.52	7.72
T° (C°)	29.0	27.2	27.0	25.7	25.3	27.3	23.7	24.7	27.4	24.1	28.0	27.4
O_2 (ppm)	4.72	3.67	2.95	4.84	4.82	4.79	4.91	5.93	5.6	5.23	5.17	4.74

Table 2

Table 2. List of CECs identified by LC-HRM, including the detection method. detection frequency and predicted ecotoxicity.

Name	CAS	Main applications	Polarity	Workflow	Products qualified in All Ions	Detection Frequency (%)	Predicted EC_{50} (mol L^{-1}) *
3.5-Diiodotyrosine	300-39-0	Natural product	Positive	All Ions	2	50	4.56E-06
4-Chlorosalicylic acid	5106-98-9	Pharmaceutical intermediate/several uses	Negative	All Ions	2	33	2.82E-04
4-Nitrophenol	100-02-7	Several uses	Negative	Auto MSMS	-	100	2.07E-03
8-Chlorotheophylline	85-18-7	Pharmaceutical (antiemetic)	Negative	Auto MSMS	-	75	2.75E-07
8-Hydroxyefavirenz	205754-33-2	Metabolite of efavirenz	Negative	Auto MSMS	-	100	9.75E-05
8-Hydroxyquinoline	148-24-3	Natural product/several uses	Positive	All Ions	2	75	5.93E-04
Acetaminophen	103-90-2	Pharmaceutical (analgesic)	Positive	Auto MSMS	-	58	7.93E-04
Acetanilide	103-84-4	Pharmaceutical (analgesic)/several uses	Positive	Auto MSMS	-	42	2.85E-04
Amantadine	768-94-5	Pharmaceutical (Parkinson treatment)	Positive	All Ions	2	92	2.00E-04
Amezinium	30578-37-1	Pharmaceutical (hyperthension treatment)	Positive	All Ions	2	100	2.15E-04
Atenolol	29122-68-7	Pharmaceutical (betha-blocker)	Positive	Auto MSMS	-	92	8.30E-05
Azelaic acid	123-99-9	Natural product / plasticizer metabolite	Positive	Auto MSMS	-	8	8.28E-06
Benzoylecgonine	519-09-5	Metabolite of cocaine (drug of abuse)	Positive	Auto MSMS	-	100	2.28E-06
Betaxolol	63659-18-7	Pharmaceutical (betha-blocker)	Positive	All Ions	2	92	1.85E-06
Butylbenzylphthalate	85-68-7	Plasticizer	Positive	Auto MSMS	-	100	2.59E-03
Caffeine	58-08-2	Stimulant	Positive	All Ions	8	100	3.65E-06
Carbamazepine	298-46-4	Pharmaceutical (anticonvulsant)	Positive	All Ions	2	58	5.96E-05
Carbendazim	10605-21-7	Funguicide	Positive	Auto MSMS	-	100	0.00013 (1)
Clarithromycin	81103-11-9	Pharmaceutical (antibiotic)	Positive	All Ions	6	50	2.04E-04
Crotetamide	6168-76-9	Pharmaceutical (analgesic)	Positive	All Ions	2	100	1.22E-04
Dexpanthenol	81-13-0	Provitamin/pcp	Positive/Negative	All Ions	8	92	5.58E-03

Name	CAS	Main applications	Polarity	Workflow	Products qualified in All Ions	Detection Frequency (%)	Predicted EC$_{50}$ (mol L^{-1}) *
Diclofenac	15307-86-5	Pharmaceutical (antiinflamatory)	Negative	Auto MSMS	-	33	1.36E-06
Diethyltoluamide	134-62-3	Insect repellent	Positive	Auto MSMS	-	100	7.40E-06
Dinoterb	1420-07-1	Herbicide	Negative	Auto MSMS	-	17	8.41E-05
Diuron	330-54-1	Herbicide	Positive/Negative	All Ions	3	100	1.07E-03
Ecgonine methyl ester	7143-09-1	Metabolite of cocaine (drug of abuse)	Positive	All Ions	2	75	8.67E-06
Enalapril	75847-73-3	Pharmaceutical (hyperthension treatment)	Positive	Auto MSMS	-	8	1.14E-04
Esculetin	305-01-1	Antioxidant/Natural product	Negative	Auto MSMS	-	42	7.80E-07
Fendiline	13636-18-5	Pharmaceutical (Calcium channel blocker)	Positive	All Ions	3	100	1.96E-08
Fipronil	120068-37-3	Insecticide	Negative	Auto MSMS	-	100	7.11E-04
Gabapentin	60142-96-3	Pharmaceutical (anticonvulsant)	Positive	All Ions	3	92	0.000000735 (2)
Irbesartan	138402-11-6	Pharmaceutical (hyperthension treatment)	Negative	Auto MSMS	-	92	0.000000553 (2)
Losartan	114798-26-4	Pharmaceutical (hyperthension treatment)	Positive	Auto MSMS	-	42	0.214 (2)
Metformin	657-24-9	Pharmaceutical (antidiabetic)	Positive	All Ions	8	100	2.94E-06
Methoprene	40596-69-8	Insecticide	Positive	All Ions	5	67	7.01E-07
Mycophenolic acid	24280-93-1	Pharmaceutical (immunosuppresant)	Positive	Auto MSMS	-	67	2.29E-05
N-Desalkylverapamil	34245-14-2	Metabolite of verapamil (hypertension treatment)	Positive	All Ions	5	67	1.47E-04
Octhilinone	26530-20-1	Funguicide	Positive	Auto MSMS	-	75	2.69E-06
Oxeladin	468-61-1	Pharmaceutical (cough suppressant)	Positive	All Ions	3	100	2.67E-05
Palmidrol	544-31-0	Natural product/supplement	Positive	All Ions	2	100	5.96E-06
Paraxanthine	611-59-6	Metabolite of caffeine (stimulant)	Positive	Auto MSMS	-	100	1.03E-02
Phenethylamine	64-04-0	Natural product/several uses	Positive	All Ions	5	33	3.88E-04
Piperine	94-62-2	Natrual product/Pharmaceutical (diuretic)	Positive	Auto MSMS	-	83	1.55E-06

Name	CAS	Main applications	Polarity	Workflow	Products qualified in All Ions	Detection Frequency (%)	Predicted EC_{50} (mol L^{-1}) *
Riboflavin	83-88-5	Vitamin	Positive	All Ions	2	50	1.70E-04
Ritonavir	155213-67-5	Pharmaceutical (antiretroviral)	Positive	All Ions	3	25	0.000000108 (2)
Salicylic acid	69-72-7	Pharmaceutical (antiinflamatory)	Negative	Auto MSMS	-	92	7.08E-04
Sebuthylazine or terbuthylazine	7286-69-3; 5915-41-3	Herbicide	Positive	Auto MSMS	-	100	0.0000525 or 0.000104713
Sucralose	56038-13-2	Sweetener	Negative	All Ions	1	92	1.39E-03
Terbutryn	886-50-0	Herbicide	Positive	Auto MSMS	-	100	6.44E-05
Testolactone	968-93-4	Pharmaceutical (antineoplastic)	Positive	All Ions	2	33	1.56E-05
Tetrahydrocortisone	53-05-4	Hormone	Positive	All Ions	2	33	7.24E-05
Tetramethrin	7696-12-0	Insecticide	Positive	All Ions	2	25	2.20E-07
THC-COOH (11-Nor-9-Carboxy-tetrahydrocannabinol)	56354-06-4	Metabolite of tetrahydrocannabinol (drug of abuse)	Positive	All Ions	2	42	3.33E-07
Theophylline	58-55-9	Stimulant	Negative	Auto MSMS	-	67	1.00E-02
Thymotic acid	548-51-6	Several uses	Negative	Auto MSMS	-	92	1.20E-04
trans-10,11-Dihydroxy-10,11-dihydrocarbazepine	58955-93-4	Metabolite of carbamazepine (anticonvulsant)	Positive	All Ions	3	100	8.13E-05
Triamterene	396-01-0	Pharmaceutical (diuretic)	Positive	Auto MSMS	-	92	1.36E-05
Tri-iso-butyl phosphate (TiBP)	126-71-6	Plasticizer/Flame retardant	Positive	Auto MSMS	-	100	2.70E-05
Tri-n-butyl phosphate (TnBP)	126-73-8	Plasticizer/Flame retardant	Positive	Auto MSMS	-	100	4.21E-05
Triphenyl phosphate	115-86-6	Plasticizer/Flame retardant	Positive	Auto MSMS	-	100	1.17E-06
Tris(2-butoxyethyl) phosphate (TBEP)	78-51-3	Plasticizer/Flame retardant	Positive	Auto MSMS	-	100	4.90E-05
Tris(2-chloroisopropyl) phosphate (TCPP)	13674-84-5	Plasticizer/Flame retardant	Positive	Auto MSMS	-	100	2.21E-05
Ugurol	1197-18-8	Pharmaceutical (antihemorragic)	Positive	All Ions	2	50	3.39E-04
Umbelliferone	93-35-6	Pharmaceutical/Natural product	Negative	Auto MSMS	-	100	1.49E-04
Usnic acid	7562-61-0	Natural product	Negative	All Ions	3	50	3.89E-06
Valdetamide	512-48-1	Pharmaceutical (hypnotic)	Positive	All Ions	5	42	1.42E-04

Name	**CAS**	**Main applications**	**Polarity**	**Workflow**	**Products qualified in All Ions**	**Detection Frequency (%)**	**Predicted EC$_{50}$ (mol L^{-1}) ***
Valsartan	137862-53-4	Pharmaceutical (hyperthension treatment)	Negative	Auto MSMS	-	42	0.000144 (2)
Venlafaxine	93413-69-5	Pharmaceutical (antidepressant)	Positive	All Ions	7	42	2.28E-05

* Predicted toxicity obtained for *Fathead minnow* from the US EPA Chemical Dashboard (https://comptox.epa.gov/dashboard) and produced by the US EPA TEST software. excepting:

(1) Obtained also from the Chemical Dashboard but for *Japanese medaka* (not available for *Fathead minnow*)

(2) Data for *Fathead minnow* predicted by ECOSAR since Chemical Dashboard did not contain such data.