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The risk of bacterial virulence in the face of concentrated river pollution

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ABSTRACT

The decrease in low-water flows and the increase in water temperature and other parameters as observed in the rivers over the last 50 years suggest that a concentration of compounds and pollutants is taking place, in connection with climate change and/or anthropisation (without discerning their respective contributions). These effects occur in a context where the rivers are already impacted by the presence of many pollutant cocktails (pesticides, drugs, and others). The authors now show that these pollutant cocktails – at the environmental concentrations currently found – constitute a threat to human health through their possible effects on the virulence of pathogenic bacteria. While certain genes of *Salmonella* Typhimurium may not experience an increased risk, the exposure to more concentrated cocktails (at a five-fold concentration) could potentially amplify certain virulent factors such as the motility of *Pseudomonas aeruginosa* H103. The findings indicate that pollution mixtures have an effect on the virulence potential of certain waterborne pathogenic bacteria, even at concentrations currently observed in rivers.

Key words: bacteria, climate change, cocktail, pollutants, virulence

HIGHLIGHTS

- The 'concentration effect' of pollutants, due to climate change, has consequences in rivers and on the virulence of pathogenic bacteria.
- There is a link between pollutants and infectious diseases.
- The presence of organic contaminants in rivers can exacerbate or not the virulence of bacteria.

1. INTRODUCTION

In a recurrent context of decreasing river flows and earlier, more severe and longer low-water periods, and conflicts for the water resources are becoming increasingly frequent. According to the Intergovernmental Panel on Climate Change projections (IPCC), beyond the 2 °C of warming in reference to 1990, each additional degree could lead to a reduction in the renewable water resources of 20% for at least 7% of the world's population. This preoccupying situation jeopardises the fragile balance between the use of water for socio-economic purposes and the preservation of aquatic ecosystems. In France, surface waters are strongly solicited in summer (when the water is already naturally at its lowest level) for drinking water production, agricultural irrigation, and the cooling of nuclear power plants. Further to the difficulties that are associated with water scarcity, the quality of water could also possibly deteriorate with climate change. Indeed, chemical pollutants, exogenous microbial flora from treated wastewaters, animal faeces, and manure, together with all of the organic or mineral components that are naturally present in the water, will be concentrated by the decreasing river flows (Figueroa *et al.* 2016; Rodríguez *et al.* 2018).

This 'concentration effect' raises concerns in terms of the water quality for human health or aquatic fauna. Chemical pollutants are frightening because of their toxicity and long-term effects but they can also act indirectly on living organisms. In fact, new concepts are emerging that point to possible links between pollutants and infectious diseases (Hodges & Tomcej 2016). Chemicals may also stimulate pathogens and, in some cases, may increase their virulence. Low levels of exposure to dioxin have resulted in increased mortality due to the influenza virus (Burleson *et al.* 1986). Lin *et al.* (2013) have shown that phthalates (DEHP) increase the cytotoxicity of *Helicobacter pylori*. Correlations have been found between

exposure to high molecular weight phthalates and the higher risks of nasal colonisation by *Staphylococcus aureus* or between fluorinated compound (PFAS) exposure and respiratory infections, or gastroenteritis (Impinen *et al.* 2019). More generally, pollutants are also being blamed for their effect on the immune response and the reduced effectiveness of vaccines (Konkel 2016; Grandjean *et al.* 2017).

In the environment, the non-deleterious effects of bacterial exposure to pollutants are (mostly) studied exclusively from the perspective of microbial resistance. Undoubtedly, the presence of antibiotics, as well as other compounds, is suspected to contribute to the maintenance or emergence of resistance genes, making certain bacterial infections difficult or impossible to treat (Davies & Davies 2010). Pollutants can also stimulate biofilm formation, quorum sensing, or toxin production, which are bacterial virulent factors that facilitate the infection of the hosts (Driscoll *et al.* 2007; Vandeputte *et al.* 2011; Lee & Zhang 2015). Therefore, the present work has aimed to determine whether the presence of organic contaminants in the rivers exacerbates the virulence of pathogenic bacteria and whether the concentration effect could increase this risk. For this work, different sites in a small French river were sampled and extracts were made up to isolate the cocktails of pollutants and compounds. These cocktails were tested for their impact on two bacterial virulent strains (*Pseudomonas aeruginosa* H103, *Salmonella enterica* Typhimurium) and on the wild-type of *Escherichia coli* K12.

2. MATERIALS AND METHODS

2.1. Watershed description and sampling

The River Clain is a tributary of the River Vienne, itself a tributary of the River Loire. It is typical of the many small rivers found in the plains of France. Its drainage basin extends over 2,882 km^2, with essentially agricultural land (90% of the surface) for cereal cultivation and mixed farming-livestock areas. The economic and industrial activities are mainly concentrated in the Poitiers agglomeration (90,000 inhabitants). Its watershed is subject to an oceanic climate, hot and dry in the summer, mild, and humid in the winter. The River Clain has a rainy regime, with high water in the winter (peak flow in January, with an average monthly flow of 27 m^3/s in Poitiers) and a low-water period in the summer from August to September.

Ten sites along the River Clain watershed were sampled in July 2020, from the source to the outlet (the confluence with the River Vienne): Pressac amont (1 km), Pressac (5 km), Verneuil (12 km), La Touche (22 km), Sommières du Clain (40 km), amont Vonne (70 km), Avenue de Lorch (90 km), Moulin apparent (99 km), La Folie (102 km), and Naintré (126 km). More details on the position of the sampling sites in the catchment area are presented in the Supporting Information (Table SI 1 and Figure SI 1). Briefly, at each sampling site, 2 L of water was sampled in glass containers and stored at 4 °C on the sampling day. The water samples were first pre-filtered by a 0.45 μm PVDF membrane (Durapore®, Merck KGaA, Darmstadt, Germany) and then prepared for further analyses.

2.2. Micropollutant analysis

2.2.1. Chemical reagents

A total of 47 compounds were selected as representative micropollutants, as commonly detected in the studied areas. The selected micropollutants included pharmaceutical compounds and pesticides, classified by their functional groups (antibiotic, anti-inflammatory, anti-epileptic, antihistamine, antiparasitic, beta-blocker, and others), and categorised by the target organisms (herbicides, insecticides, and fungicides). All of the compounds were purchased from Sigma Aldrich (Steinheim, Germany) in powder form. The stock solutions of the individual compounds were prepared in methanol at 200 mg/L and then stored at −20 °C. Several mixed standard solutions were prepared in methanol by a dilution of the individual solutions. The concentrations of the compounds in these mix solutions were 10 mg/L for each compound and they were stored at 4 °C.

The ultra-pure water (UPW) with 18.2 MΩ cm resistivities was obtained by using a Milli-Q® system (Milli-Q® IQ 7000, Merck KGaA, Darmstadt, Germany). Methanol (MeOH), acetonitrile (ACN), acetic acid, and formic acid were LCMS grade and they were purchased from Sigma Aldrich.

2.2.2. Extraction procedure

The extraction procedure was done according to Aubertheau *et al.* (2016) and Zind *et al.* (2021). For each site, a 500 mL water sample was extracted by solid-phase extraction (SPE) on an Autotrace™ 150 (Thermo Scientific Inc., Waltham, USA) (APHA 2017). Briefly, Oasis HLB cartridges (6 cubic centimetre '6cc') that were purchased from Waters Corporation (MA, USA) were conditioned with 5 mL MeOH, followed by 5 mL UPW. The water sample (500 mL) was loaded at a

constant flow rate of 10 mL/min. The cartridges were then washed with 5 mL of UPW, dried under a nitrogen flow for 5 min at 10 mL/min, and stored at −80 °C until analysis. The target compounds were eluted from the cartridges with 8 mL of MeOH and then concentrated to dryness under a gentle stream of nitrogen at 30 °C when using a TurboVap® LV system (Biotage AB, Uppsala, Sweden). The dry residues were reconstituted in 500 µL of MeOH/UPW (10/90; v/v) and spiked with increasing concentrations of a mixture of analytical standards (final concentrations of 0; 0.01; 0.05, and 0.1 mg/L) for quantification by the standard addition method. Prior to the analyses, the prepared extracts were filtered by a 0.2 µm PVDF Mini-UniPrep™ (Whatman™).

2.2.3. LC-MS analysis

The pharmaceutical compounds were separated by ultra-high-performance liquid chromatography (UHPLC, Nexera X2, Shimadzu France SAS, Marne la Vallée, France) on an ACQUITY UPLC™ BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters Corporation, Milford, USA) and maintained at 25 °C. A flow rate of 0.4 mL/min and an injection volume of 5 µL were used, with the mobile phase composed of UPW and MeOH, both of which were acidified with 0.1% of formic acid.

The pesticides were separated by a UHPLC Acclaim™ Rapid Separation Liquid Chromatography (RSLC) C18 column (2.1 × 100 mm, 2.2 µm; Thermo Fisher™ Scientific) and maintained at 25 °C. A flow rate of 0.3 mL/min and an injection volume of 5 µL were used, with the mobile phase composed of UPW that was acidified with 0.04% of acetic acid, together with ACN that was acidified with 0.1% of formic acid. The chromatography gradient for both methods is detailed in the Supporting Information (Tables SI 2 and SI 3).

The LC-MS/MS analyses were carried out on a triple quadrupole mass spectrometer (LCMS-8060, Shimadzu France SAS, Marne la Vallée, France) that was equipped with an electrospray ionisation source (ESI) while operating in a multiple reaction monitoring (MRM) mode. A total of 17 pesticides and a collection of 32 pharmaceutical compounds were chosen as reference standards to be examined in the samples. The retention times and the MRM transitions of the pesticides and pharmaceutical compounds are presented in the Supporting Information (Tables SI 4 and SI 5). Further details regarding the method validation (linearity, limits of detection and quantification, repeatability, and reproducibility) are presented in the Supporting Information (SI 6, Tables SI 7 to SI 10).

2.2.4. Agglomerative hierarchical clustering

Agglomerative hierarchical clustering (AHC) is an iterative classification method that works from the dissimilarities between the objects to be grouped. Two objects, which when clustered together, minimise a given agglomeration criterion, thus creating a class comprising of the two objects. The dissimilarity between this class and the N-2 other objects is then calculated using the agglomeration criterion. The two objects, or the classes of objects, whose clustering together minimises the agglomeration criterion, are then clustered together. This process continues until all of the objects have been clustered. This clustering was applied to determine whether the classes could be identified based on the pharmaceutical compound concentrations or the pesticide concentrations. The AHC was realised when using a similarity matrix (Pearson's coefficient of correlation) and the Simple linkage agglomeration method (XLStat-Basic add-in for Microsoft Excel™).

2.3. Virulence analysis

2.3.1. Preparation of the pollutant cocktail extracts

A 500 mL water sample was extracted by SPE on an Autotrace™ 150 (Thermo Scientific Inc., Waltham, USA). Briefly, Oasis HLB cartridges (6 cc) that were purchased from Waters Corporation (MA, USA) were conditioned with 5 mL MeOH, followed by 5 mL UPW. The water sample (500 mL) was loaded at a constant flow rate of 10 mL/min. The cartridges were then washed with 5 mL of UPW, dried under a nitrogen flow for 5 min at 10 mL/min, and stored at −80 °C until analysis. The cartridges were eluted with 8 mL of MeOH and then concentrated to dryness under a gentle stream of nitrogen at 30 °C when using a TurboVap® LV system (Biotage AB, Uppsala, Sweden). The extracts were reconstituted in 1 mL of MeOH and stored at −20 °C for further analysis.

2.3.2. Bacterial strains and culture conditions

Three bacterial strains were studied: *E. coli* K12, from the EBI laboratory collection, *P. aeruginosa* H103, which was isolated from patients with cystic fibrosis (Hancock *et al.* 1983), and *S.* Typhimurium, which was purchased from ATCC (American Type Culture Collection): ATCC® 14028™. All of the strains were maintained in a Lysogeny broth medium (LB) (1% Tryptone, 0.5% yeast extract, and 1% NaCl, BIOKAR Diagnostics) containing 30% glycerol (vol/vol) (Sigma Chemical) at −80 °C.

The bacteria were pre-cultured by transferring a typical colony on a Petri dish containing Lysogeny broth agar (LB) (1.5% agar type E), followed by incubation at 37 °C for 24 h.

2.3.3. Bacteria growth test

Each 200 μL water extract (the cocktail) was first diluted in 4 mL of LB broth. The test solutions were then prepared by using a five-fold serial dilution in LB broth: 1/5, 1/25, corresponding to the real environmental concentration, and 1/125, in polypropylene conical tubes (15 mL, SARSTED). The three bacteria strains were diluted in LB broth to yield an inoculum of approximately 10^6 CFU/mL, as determined by an optical density at 600 nm when using a Jenway 6320D spectrophotometer. The bacterial inocula were then inoculated into the test solutions (1/5, 1/25, and 1/125), with 5% methanol in a 96-well plate (Nunclon™ Delta Surface, Thermo Scientific). The growth was measured after 24 h of incubation at 37 °C, by an optical density at 595 nm when using an Infinite® M Plex microplate reader (Tecan, Switzerland). The percentages of growth were calculated relative to the control cultures of each strain (5% MeOH) after 24 h of incubation. All of the measurements were performed in triplicate. The results of each concentration were statically analysed against control (5% MeOH) to determine their significance, by using one-way ANOVA, followed by Dunnett's post hoc test.

2.3.4. Motility assay

The swarming motility assay was performed for the three bacteria strains by stab inoculating the centre of the LB solid medium (0.6% agar), with the bacteria suspension (control, in 5% MeOH, 1/25, the real environmental concentration, and 1/5, the real × 5) at room temperature. The plates were covered and left to stand for 10 min at room temperature, and then they were placed upside-down in the incubator at 37 °C for 24 h (*P. aeruginosa* H103), or 48 h (*E. coli* K12 and *S.* Typhimurium).

2.3.5. Relative expression of the virulent genes

P. aeruginosa H103 and *S.* Typhimurium were cultivated in 1/25 and 1/5 test solutions by using four water extracts (namely, the sites at 1, 12, 40, and 102 km) at 37 °C in 4 mL of LB broth for 24 h. The cultures were then centrifuged at 5,000 g for 10 min at 4 °C. After discarding the supernatant and removing any remaining media by aspiration, the bacteria pellets were kept at −80 °C for the mRNA extraction.

2.3.6. RNA extraction

An RNeasy Mini Kit (QIAGEN) was used to extract the mRNA from the *P. aeruginosa* H103 and *S.* Typhimurium cultures. The bacteria envelopes were destroyed by incubation at 37 °C for 10 min after adding 8 μL of lysosome (50 mg/mL). The mRNA samples were then processed by following the manufacturer's protocol.

2.3.7. Real-time Polymerase Chain Reaction (PCR)

The GoScript™ Reverse Transcription kit (Promega) was used for the cDNA synthesis according to the manufacturer's recommendations. The prepared mixture containing 4 μg of DNase-treated RNA and 1 μg of Primer oligo dT/Random primer (1:1) was heated at 70 °C for 5 min, and then chilled on ice for 5 min. 15 μL of RT-PCR mix reaction was then added to each sample. The cycle conditions were as follows: 5 min at 25 °C, followed by 60 min at 42 °C, and then with RT inactivation for 15 min at 70 °C. The resulting cDNA samples were set at −20 °C.

2.3.8. Quantitative PCR

The *P. aeruginosa* H103 sequence of *algD* (resistance to the immune system), *exoS* (acute infection), *lasI* (formation of biofilm), and the *oprL* (outer membrane protein, reference) genes, together with the *S.* Typhimurium sequence of *invA* (invading the epithelial cells), as well as the *stn* (production of enterotoxin) and *rss16S* (reference) genes, were all quantified when using the primers (the sequences are presented in Table SI 11) that were purchased from the Eurogentec partnership. The quantitative PCR (qPCR) was designed to amplify the bacteria genes when using Takyon™ SYBR® 2X qPCR MasterMix Blue (Eurogentec) and the LightCycler®480 system (Roche Applied Science, Germany). The reaction mixture contained 2.4 μL of UPW, 5 μL of 2X Takyon™ MasterMix, 0.3 μL of each primer (10 μM), and 2 μL of the diluted cDNA template, to form a final volume of 10 μL. Negative control was performed to examine any genomic DNA contamination. The qPCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s, 57 °C for 10 s (for all of the studied genes except *oprL* and *rss16S*, for which the specific temperatures were 51 and 60 °C, respectively), and 72 °C for 10 s. A melting curve analysis ranging from 65 to 95 °C was used, to specify the amplicon for each primer pair.

The determination of gene expressions was performed in triplicate. The number of gene copies was calculated by using a constructed standard curve. The quantitative variation between the replicates was evaluated when using the $2^{-\Delta\Delta CT}$ relative quantification method (Livak & Schmittgen 2001). The relative expression of the *algD*, *lasI*, and *exoS* genes was normalised to the *oprL* gene, whereas *stn* and *invA* were normalised to the *rss16S* gene.

2.4. Statistical analysis

A one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test (GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California, USA, www.graphpad.com), was performed to ensure the significance of the bacteria growth results. The optical density of the control was compared to that of the tested solutions for each bacteria strain. The statistical analysis was performed on the ΔCt values (Livak & Schmittgen 2001). An unpaired *t*-test was performed to determine whether the differences between the two test solutions of the gene expressions were significant. All of the test results were significant when the *p* values were <0.05 ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

3. RESULTS AND DISCUSSION

3.1. Was there already evidence of a concentration effect?

The main data that were available to observe the visible impacts of climate change on the surface water quality was essentially limited to the global parameters, such as temperature or practical salinity. These parameters have been monitored for several decades and the acquisition methods have evolved relatively little. In France, such data have been collected, banked, and made accessible by the Water Agencies (created in 1964) (JORF 1964).

For the River Clain, the temperature, salinity, and flow rate have been recorded at the Dissay Monitoring Station, located at 122 km from the source since the 1960s. The data from this station have suggested a trend of 1.2 °C increase in the water temperature over the last 50 years (1971–2021) (Figure 1). This trend is in agreement with studies on other French basins, such as the River Rhone basin, which predicted an average temperature increase of 2.1 °C from 2071 to 2100 (when compared to 1971–2000), according to the SRES A2 and B1 scenarios (Aubé 2016). These studies have estimated that 79% of the increase in the maximum temperature is due to the increase in air temperature, and 21% to the decrease of inflow. More than 90% of the increase in the average temperatures would be due to the increase in air temperatures (van Vliet *et al.* 2013). The Explore 2070 project (MEED 2012) has predicted that the increase in French river temperatures between the periods 1961–1990 and 2046–2065 will be between 1.09 and 2.16 °C (with an average of 1.64 °C). To be clear, each river is not likely to be affected in the same way. The temperatures of large rivers are primarily controlled by atmospheric conditions, while the temperatures of the small and medium-sized rivers may depend on the geographical and geomorphological conditions.

The conductivity data of the River Clain have also shown a slightly increasing trend of 71 µS/cm between 1971 and 2021 (Figure 1). Such changes in the water quality parameters have also been observed for other French river basins, such as the River Loire, the River Rhône, and the River Seine (Aubé 2016). Nonetheless, the responsibility of climate change in these increases is considered minor when compared to the direct anthropogenic activities (Pachauri & Reisinger 2007; Floury *et al.* 2013). For the River Loire, the decrease of inflows of approximately -100 m^3/s, and the increase in the average annual water temperature of $+1.2$ °C ($+2$ °C in summer) only explains 20% of the variation in the quality parameters for the period 1977–2008 (Aubé 2016). The River Clain watershed accounts for 60% of the county's population. In this county, the population density has increased from 51.1 to 62.5 inhabitants/km² between 1975 and 2017 (INSEE 2021) while also showing a slight increase in anthropisation.

Several studies that have been conducted in France predict a decrease in the summer flows (for example, 46% for the River Rhône Mediterranean basins (van Vliet *et al.* 2013; Giuntoli *et al.* 2015)), with a consequent increase in the severity of low-flows (Ruiz-Villanueva *et al.* 2015), hence the concentration of pollutants (organic or mineral) in the water. A study of River Clain data (Figure 1) also suggests a slight decrease in the low-flow rate between 1965 and 2020. According to linear regression, the flow decreased by 1.35 m^3/s over the period. Even so, it is difficult to attribute this decrease solely to climate change because of the evolution of water withdrawals by agriculture (crop irrigation), or by the population, which has also increased during this same period (Aubé 2016). It should be noted that the value of the low-flow objective (=the average monthly flow above which it is considered that all uses are possible, in balance with the proper functioning of the aquatic environment) is 3 m^3/s for the River Clain. This threshold has been exceeded (i.e. lower values) 11 times since 1965.

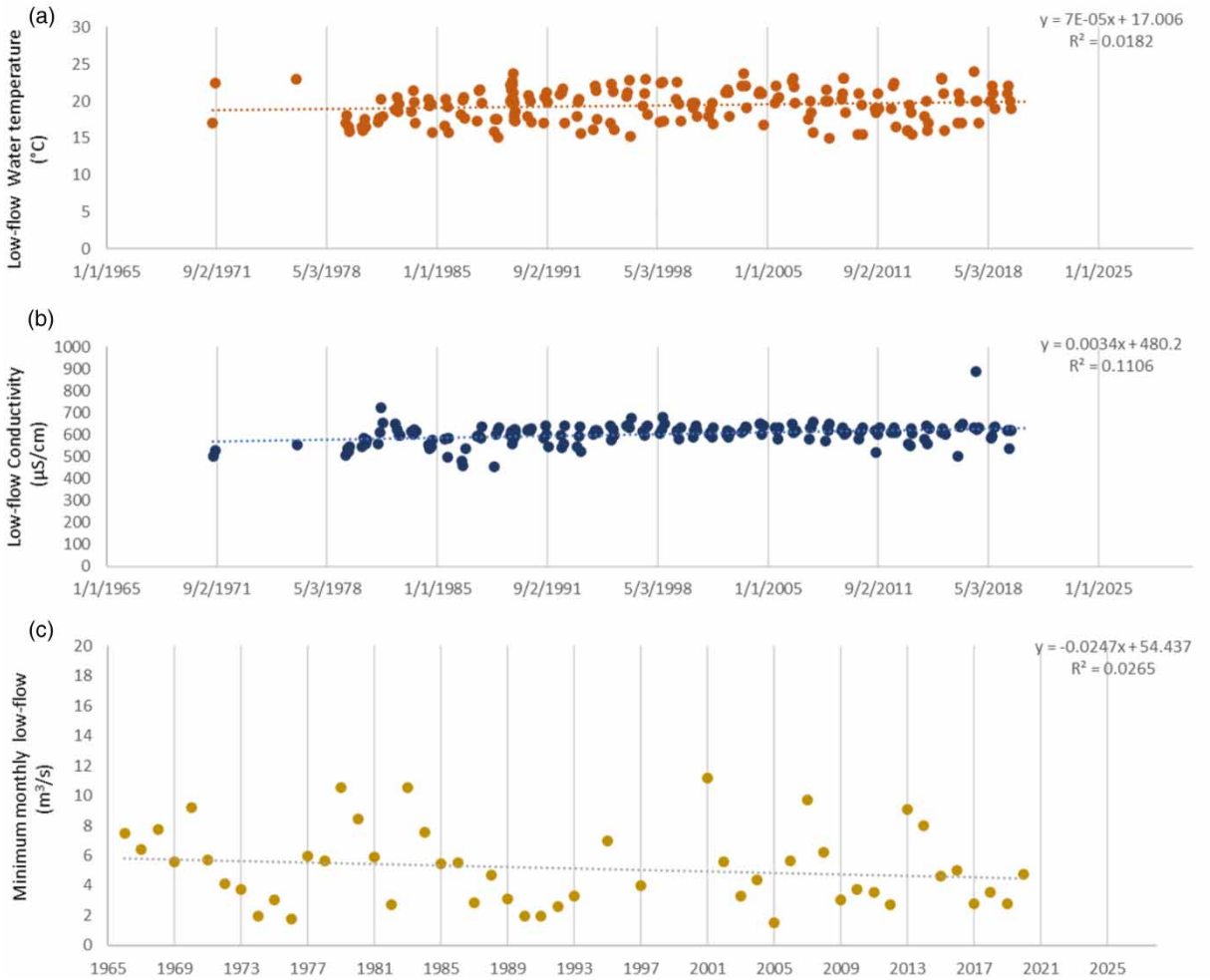

Figure 1 | Water temperature (a) and conductivity (b) of the River Clain from 1965 to 2021, during the low-flow period (June, July, August, September). The values correspond to the daily data that were obtained from the Dissay Monitoring Station (code n°04085000 – L2501610) on the River Clain. The water temperature (Sandre code n°1301) and conductivity (Sandre code n°1303) for the station were obtained from the Naïades database (http://www.naiades.eaufrance.fr/). The QMNA (c) of the River Clain for the 1965–2021 period and the QMNA was the minimum monthly low-flow for a given year. The values were obtained from the Hydro Eau France Data Bank (http://www.hydro.eaufrance.fr/). All of the values were considered except for the three outlier values of low-flow: 37.53–35.81–31.31 m^3/s, for 1998, 1999, and 2000, respectively.

3.2. Current river pollution is a huge cocktail of pollutants

Nowadays, many organic and inorganic pollutants are found in the rivers. Together, they form a cocktail, whose properties/effects differ from the individual properties or the effect of each compound (OECD 2019). This cocktail differs from site to site and evolves along the watershed. To get a first idea of the diversity of this cocktail for the organic pollutants in the River Clain, the pesticides used in agriculture, or the drugs used in veterinary or human medicine in the watershed, were quantified by targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although far from exhaustive, the list of targeted compounds nevertheless highlights the omnipresence of numerous pesticides and drug residues throughout the watershed. Many compounds were detected in 100% of the samples, from the source to the River Clain outlet (Figure 2).

Agricultural activities and the presence of numerous small and medium-sized farms and to a lesser extent, the urbanised areas and wastewater treatment plant discharges (wastewater and stormwater) explain the pollution encountered in the River Clain. Agriculture in the River Clain watershed is characterised by cereal production in the northern half (downstream) of the basin and mixed farming and polyculture in the southern half (upstream) of the basin. The dominant crops are cereals (41% of the surface area), forage (27%), and grain corn (11%) (SAGE Clain 2011). The origin of the agricultural discharges is now double: diffuse pollution

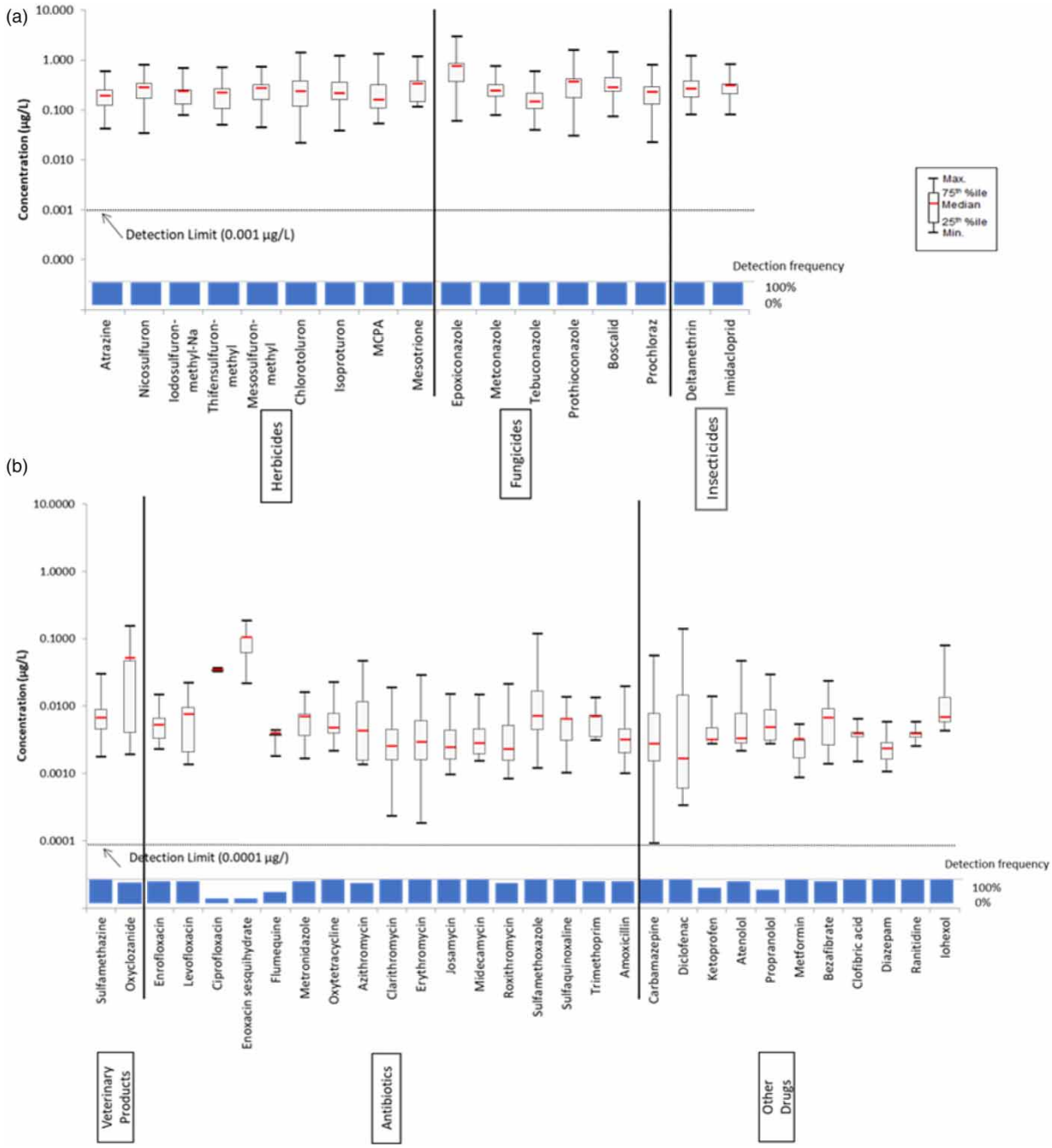

Figure 2 | Box plots and detection frequencies of pesticides (a) and drugs (b) in the water samples when considering the different sampling sites of the River Clain ($n = 10$). The box plots represent median, minimum, and maximum concentrations of the targeted compounds.

(fertilisation and use of phytosanitary products) and point source pollution (discharges from livestock buildings, accidents during the use of phytosanitary products). Urban discharges are mainly provided by the 192 wastewater treatment plants, representing a capacity of 336,299 Population Equivalent, including 24 with more than 2,000 Population Equivalent (SAGE Clain 2011).

The pesticide concentrations varied from 0.002 to 1.69 µg/L and all of the families that were searched for, such as herbicides, fungicides, and insecticides, were detected (Figure 2). As expected, the compounds and their amounts differed between the sites. These differences were illustrated by the hierarchical cluster analysis (HCA) of the data set (Figure 3). At 1 km from the source, the pesticide concentrations were low (<0.2 µg/L), and the most concentrated pesticide was the fungicide

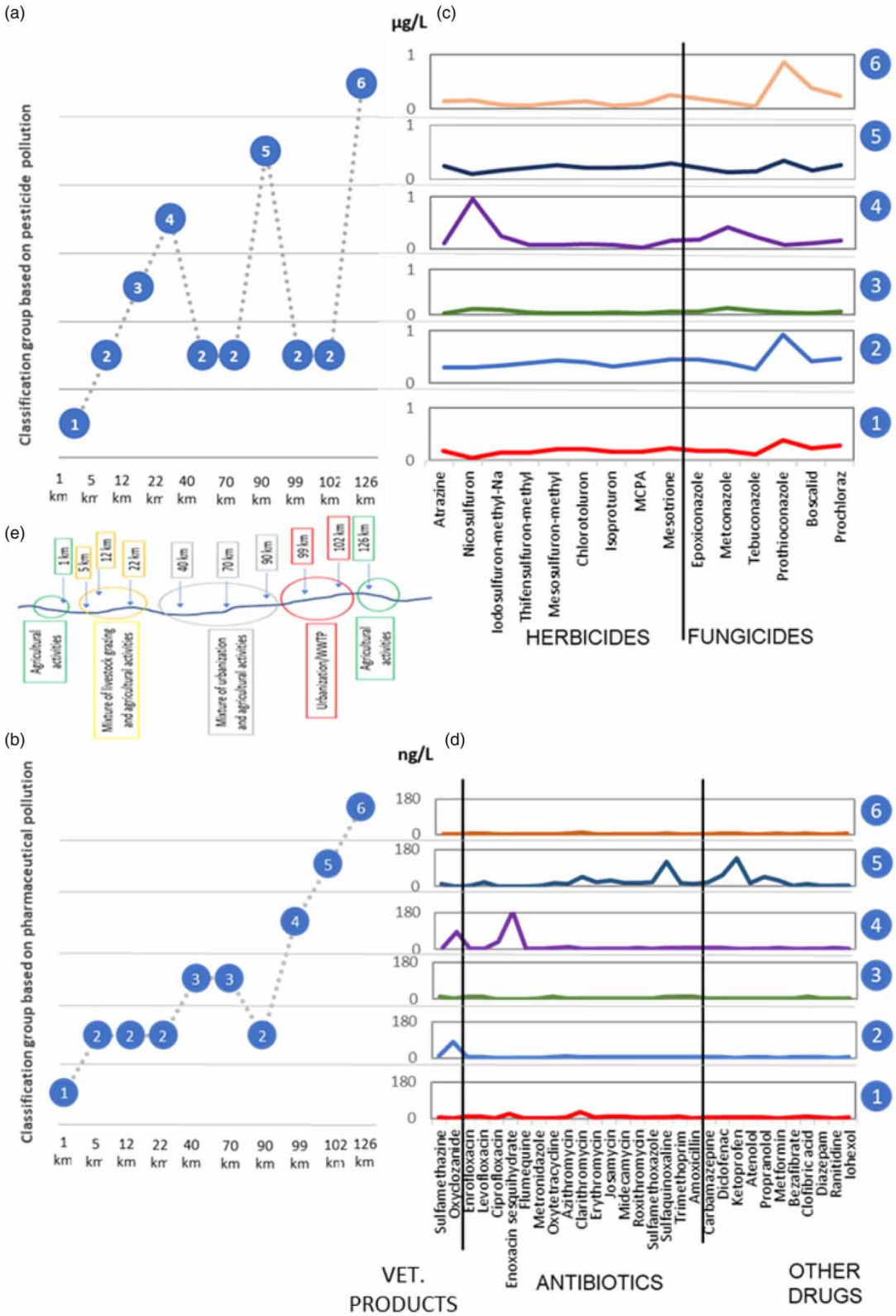

Figure 3 | Overview of the pesticides and drug pollution in the River Clain watershed. (a,b) show the assignment of each sampled site to one of the classes, as defined by the AHC (1–6), for the pesticides and drugs, respectively. (c,d) show the concentration profiles of the classes, as defined by the AHC (each curve represents the barycentre of its class), for the pesticides and drugs, respectively. (e) shows the profile of the River Clain watershed, with the main human activities. The AHC was conducted with six levels, with a 95% cut-off.

prothioconazole. At 5 km, the concentration of prothioconazole (0.51 μg/L) and the other fungicides (epoxiconazole, tebuconazole, and metconazole) increased significantly. This contamination over the first few kilometres of the River Clain is explained by the omnipresence of drained agricultural fields and farms within the catchment area. At 12 km away from the source, the pesticide concentrations decreased strongly, as the cereal crops were replaced by livestock (mainly sheep, and cows). After 22 km, the pesticide concentrations increased substantially and the presence of nicosulfuron (a herbicide) and metconazole (a fungicide) became very significant. At 40, 70, 99 and 102 km, the contamination profiles became similar to those that were observed at 5 km, with a large presence of most of the compounds but especially prothioconazole. At the outlet (126 km), the pollution of the River Clain was characterised by the presence of prothioconazole and mesotrione, in higher concentrations than the other pesticides.

The HCA analysis of the data of the drug (Figure 3) has shown that at 1 km, the drug concentrations were low (<11 ng/L, except for enoxacin = 21.44 ng/L, and azithromycin = 29.56 ng/L). At 5, 12, 22, and 90 km, the main compound detected was oxyclozanide, an anthelmintic that is used in the treatment and control of parasitosis in domestic ruminants, such as cattle, sheep, and goats. Its presence was consistent with the many livestock farms in this area of the watershed. At 40 and 70 km, the concentrations of veterinary and human drugs were very low, due to the presence of large cultivated areas, with few habitations. Downstream of the watershed, the densification of urban areas probably explains the increase in drug levels, such as ciprofloxacin and oxyclozanide at 99 km; and azithromycin, sulfamethoxazole, diclofenac, atenolol, and iohexol at 102 km (near the Poitiers wastewater treatment plant, with Wastewater Treatment Plant (WWTP) discharge). At the watershed outlet (126 km), the drug levels decreased again, due to the distance from the city of Poitiers, and with dilution.

Although modest in size and seemingly rational in its anthropisation (for a rural region, with a few urban areas and villages), the results have shown that the River Clain nevertheless carries a multitude of pollutants, forming a unique cocktail at each of the sites monitored. This contamination has led the health authorities to prefer – as much as possible – the use of groundwater, for the production of water for human consumption. Be that as it may, the organisms and microorganisms living in the River Clain have no other choice, and they are largely exposed to these mixtures of pollutants, which can potentially disturb their metabolism and development.

3.3. Can pollution help bacteria to be more infectious?

A key concern regarding the cocktail effect is the risk that the combination of pollutants, which may be present individually in harmless concentrations, presents enhanced health risks. The authors hypothesise that in addition to presenting a risk of toxicity to humans, the exposure to these cocktails could affect certain virulent factors of bacteria that are present in the rivers, including the bacteria that are pathogenic to humans. According to the WHO, the mortality of water-associated diseases exceeds 5 million people per year in the world. Most of these diseases are related to the bacterial agents that are responsible for cholera, gastroenteritis, salmonellosis, typhoid fever, bacillary dysentery, shigellosis, or acute diarrhoea (Fenwick 2006; Cabral 2010). To evaluate this hypothesis, the authors studied the effect of cocktails, which were extracted from the River Clain, on certain virulent factors (growth, motility, and specific virulent genes) of typical pathogenic bacteria.

The results have highlighted that the exposure of *E. coli* K12, or *S.* Typhimurium, to the cocktails at (the real) environmental concentration (Figure 4), led to a significant increase in the growth of these bacteria (~2–20%). On the contrary, the exposure of *P. aeruginosa* H103 to these cocktails caused a growth decrease of 2–25% (except for the 1 km site, for which the growth was increased by 12%). It would seem that exposure to these cocktails of pollutants and compounds could promote the growth of certain bacteria. The use of pollutants by bacteria has already been described many times. For example, it is well known that some pollutants can often serve as nutrient sources and stimulate growth (Wainwright 1999), while others are toxic to bacteria and inhibit both the growth and microbial activity (Abatenh *et al.* 2017). These effects on growth must be put into perspective since these bacteria do not multiply in the environment, even if they can survive from several days to several months in water. In the environment, *S.* Typhimurium survives for more than 6 months (Moore *et al.* 2003), *P. aeruginosa* H103 for less than 5 months (Lewenza *et al.* 2018), and *E. coli* K12 for less than 3 months (Wang & Doyle 1998).

The swarming tests (Figure 4) showed no effect of exposure of *E. coli* K12 and *S.* Typhimurium to the cocktails that were extracted from the River Clain. In contrast, *P. aeruginosa* H103 appeared to be less mobile when exposed to the same cocktails. Swarming motility is operationally defined as a rapid multicellular bacterial surface movement that is powered by rotating flagella (Wang *et al.* 2004; Rütschlin & Böttcher 2020). Motility is often associated with the virulence of human pathogens. Many species of *Pseudomonas* produce and secrete biosurfactants, which lower the surface tension of the swarm fluid and allow the swarm front to advance with less difficulty (Kearns 2010; Yang *et al.* 2017). This decrease in

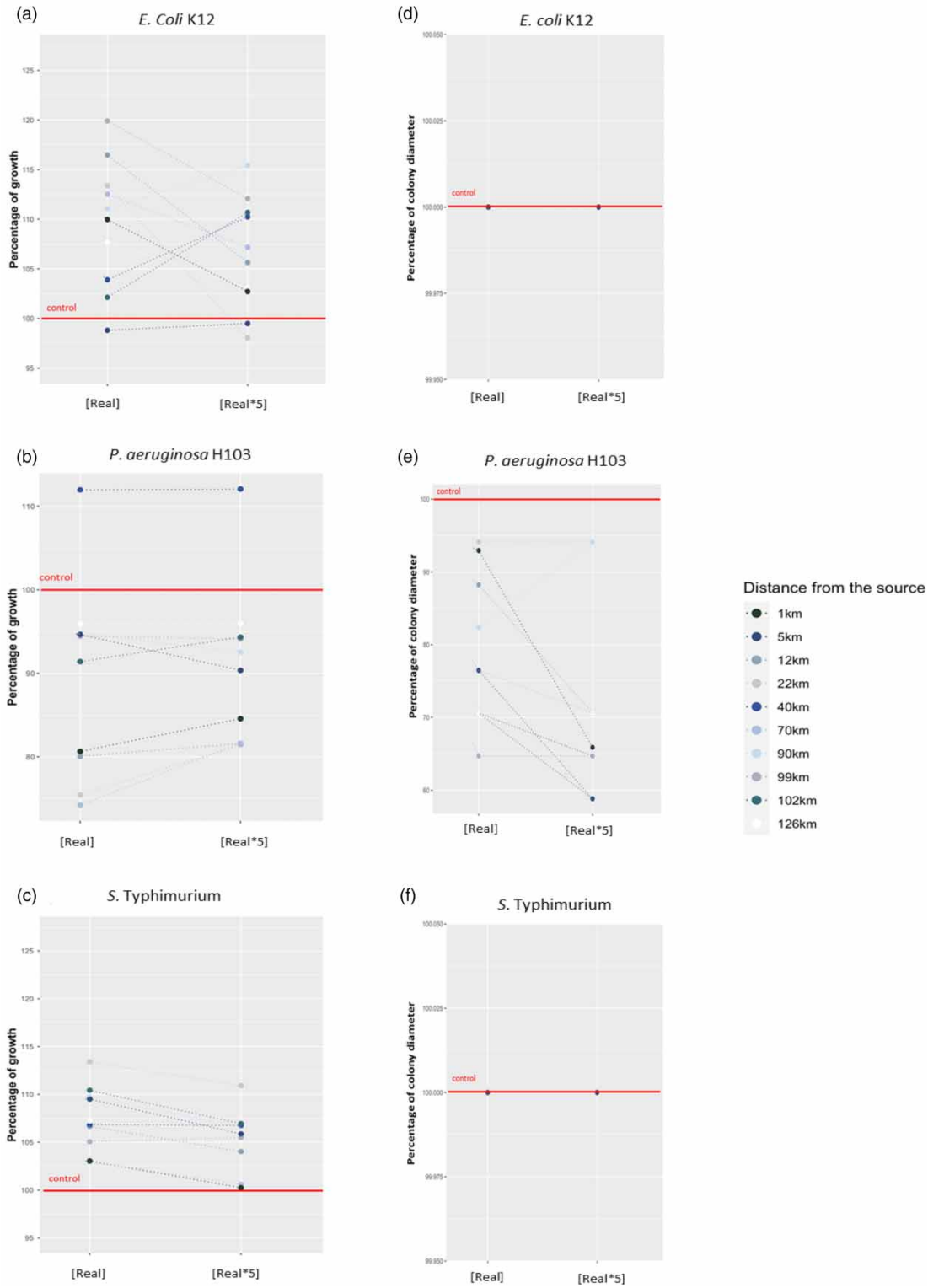

Figure 4 | Effect of the cocktail concentration on the bacterial growth (left; a–c), and the swarming motility (right; d–f), of the three studied strains: *E. coli* K12, *P. aeruginosa* H103, and *S.* Typhimurium. Three) concentrations were tested: the environmental concentration (real), five-fold the environmental concentration (real ×5), and the environmental concentration diluted five-fold (real/5). The results were subjected to one-way aNOVA, followed by Dunnett's post hoc test (GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com). The optical density of control was compared to that of the tested concentrations for each bacterium. The standards deviation (not represented) of the growth test varied from 0.1 to 6%, while for the mobility test, the value was equal to 2.4%.

mobility could be related to a disruption of the biosurfactant production by the pollutants. The virulence of *P. aeruginosa* is enhanced under swarming conditions by the upregulation of the gene expression of the type III secretion system, as well as numerous virulent factors, including extracellular proteases, and the biosynthesis for siderophores and phenazines (Overhage *et al.* 2008; Rütschlin & Böttcher 2020). This study's results have suggested that exposure to the cocktails decreased the movement capacity of *P. aeruginosa* H103 (from −5 to −35%) but it did not increase it for the other pathogen, like *S.* Typhimurium as well for *E. coli* K12.

The expressions of the *P. aeruginosa* and *S.* Typhimurium specific virulent-related genes were also measured for the water cocktails at the four sites (1, 12, 40, and 102 km): *algD* (Resistance to the immune system), *exoS* (exotoxin), and *lasI* (formation of biofilm) for *P. aeruginosa* H103; *invA* (invading the epithelial cells) and *stn* (production of enterotoxin) for *S.* Typhimurium. The results (Figure 5) show that the exposure to the cocktails strongly decreased the expression of

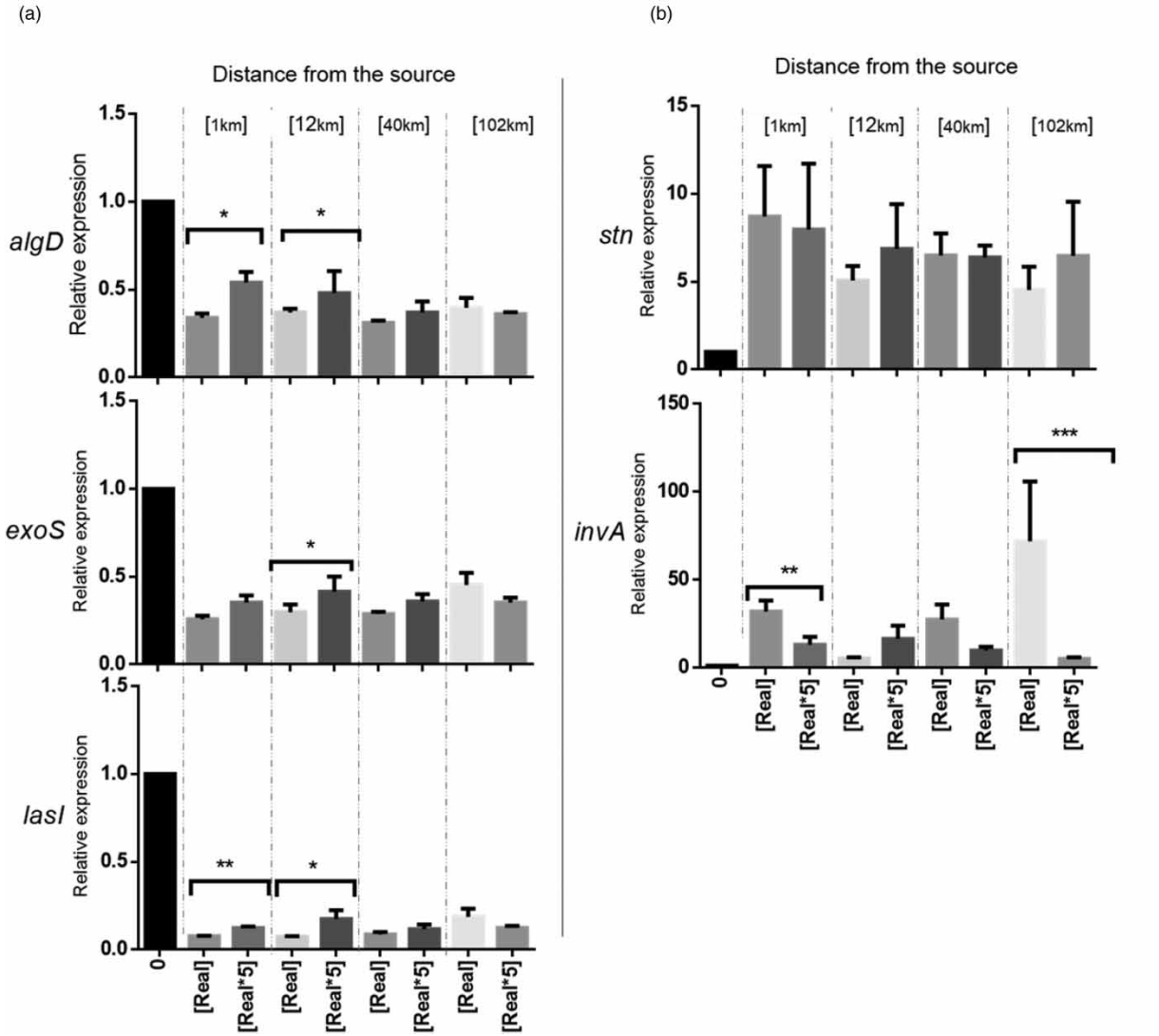

Figure 5 | The relative expression of the *algD*, *exoS*, and *lasI* genes of *P. aeruginosa* H103 (a), and the stn and *invA* genes of *S.* Typhimurium (b), at the different sites (1, 12, 40, and 102 km) for the two different concentrations of the cocktail of pollutants (real, real ×5). The relative expression of the virulent genes was compared to their reference genes (*opr*L and *rss*16S for *P. aeruginosa* H103, and *S.* Typhimurium, respectively). The results were subjected to an unpaired (independent) parametric *t*-test (GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com). For the qPCR tests, the statistical analyses were performed on the ΔCt values. All of the test results were significant when the *p* values were <0.05 ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (a) *P. aeruginosa* H103 genes expression (b) *S.* Typhimurium genes expression.

the *P. aeruginosa* H103 virulent genes (*algD*, *exoS*, and especially *lasI*) for all of the samples. Other studies have also observed that some *Pseudomonas* genes that encode metal ion transporters, drug resistance, and alginate biosynthesis were suppressed in the water when loaded with high metal concentrations (Kiliç *et al.* 2010), while the genes encoding membrane transporters, proteases, and others were overexpressed (Gómez-Sagasti *et al.* 2015; Basta *et al.* 2017). This current study's results have highlighted that exposure to the cocktails overexpressed the *S.* Typhimurium genes (5–8 fold for *stn*, and 5–60 fold for *invA*) (Figure 5). The *Salmonella* virulent genes are also known to be regulated by environmental conditions, such as the slightly alkaline pH and nutrient concentrations (Alverdy & Stern 1998; Nutt *et al.* 2003; Wang *et al.* 2004). All of these findings show that this current pollution is already having an impact on the virulent capacity of the microorganisms.

3.4. What will happen if the pollutants become more concentrated?

To evaluate the impact of the concentration of the pollutants, the same tests as above were performed with extracts that were prepared at five times their environmental concentration (real × 5). This five-fold value was chosen to show the significant changes in the virulent factors.

The exposure of *E. coli* K12 to this higher concentration increased its growth, even if it is not a considered as a pathogenic bacteria, by 5–6% for the two cocktails (sites at 40 and 102 km) but decreased it for the others (Figure 4). The growth of *S.* Typhimurium decreased slightly at the higher concentration with all of the cocktails (except for the site at 99 km). In contrast, exposure to the five-fold environmental concentration resulted in a slight increase in the growth of *P. aeruginosa* H103 for the three cocktails (22, 90, and 102 km), with no effect or a decrease for the other sites. The swarming tests (Figure 4) showed no effect of exposure to *E. coli* K12 and *S.* Typhimurium at the higher concentration, while *P. aeruginosa* H103 appeared to be even less mobile when exposed to the five-fold concentration. The study of the gene expressions (Figure 5) revealed slight changes at the higher concentrations but only for certain cocktails and certain genes. Even if *P. aeruginosa* H103 genes were underexpressed compared to the control but by increasing the concentration of the cocktails, this procedure caused either an increase in the expression of the *algD* and *lasI* genes (1 km) for *P. aeruginosa* H103, or a decrease of the *invA* gene (1, 40, and 102 km) for *S.* Typhimurium, but in fact it was overexpressed, even if gene's expression was measured after its stationary phase, where not much gene's expression activity would be expected at this point, but according to the conditions of this experiment, the studied virulent genes of *S.* Typhimurium were overexpressed.

As a consequence, several virulent factors of *S.* Typhimurium seem to be reduced in the concentration of the pollutants, notably, in its capacity to invade the host. The codes for the protein *invA* allowed the bacteria to invade the epithelial cells (Darwin & Miller 1999). Other virulent genes, from the pathogenicity islands and regulatory systems, may also be used to study to the virulence of *S.* Typhimurium such as GaDPH and Sigma 70. Other factors that are also involved in the virulence would not be overexpressed, such as growth, swarming, and enterotoxin production (*stn* gene) by the concentration effect of the pollutants. Hence, the risk of *Salmonella* infection would be reduced but it is always signalled.

On the contrary, if the concentrations did increase, some virulent factors of *P. aeruginosa* H103 could be magnified, therefore, making it more dangerous than it currently demonstrates, even if they were underexpressed but they showed an important increase compared to the lower concentration. The results have suggested that the chemical stress (induced by the cocktail) caused the expression of the genes to contribute to their pathogenicity. The expression of the *lasI* (involved in the biofilm formation and quorum sensing (Pesci *et al.* 1997; Davies *et al.* 1998) and *algD* (triggered under the conditions of slow growth or nutrient limitation) genes has shown that *P. aeruginosa* H103 was disturbed by the presence of the pollutants. Several increased genes were also involved in toxin production. The gene *lasI* also activated the transcriptional activator that was encoded by *lasR* to drive the virulent genes: *lasB*, *lasA*, *apr*, and *toxA* (Gambello & Iglewski 1991; Toder *et al.* 1991; Gambello *et al.* 1993; Pearson *et al.* 1994, 1997). The gene *exoS* was additionally involved in the secretion of exotoxin that was responsible for the acute forms of the *Pseudomonas* infection (Shaver & Hauser 2004; Fito-Boncompte *et al.* 2011). Among the cocktails studied, the magnification effect was particularly marked for the 12 and 1 km cocktails. Still, it is important to note that this effect was not observed for the other cocktails.

Similarly, the effect of exposure to a lower environmental concentration (real/5, Figure 4) was also tested. For *E. coli* K12, the results showed an even greater increase in growth at this concentration when compared to the environmental concentration. For *S.* Typhimurium and *P. aeruginosa* H103, the effect on growth was not very different (with some exceptions).

4. CONCLUSIONS

The majority of present climate predictions indicate a gradual reduction in low-water flows in the forthcoming decades. This decline, if all other factors remain constant, has the potential to result in an accumulation of compounds and pollutants in rivers. However, the impact of this 'concentration effect' will also be influenced by human activities, population dynamics, and their future evolution, which will be shaped by forthcoming political and societal decisions. Currently, the data collected from a limited catchment area, such as the River Clain in France, indicate that this process is already in progress. There is evidence of a decline in daily flow and QMNA (lowest annual flows), accompanied by an increase in conductivity. The growing concern regarding the future quality of aquatic environments is driven by the concentration of pollution. However, the findings of this study serve as a reminder that rivers are already heavily contaminated and significantly affected by the presence of pollutants. The analysis of pesticides and drugs reveals the existence of mixtures of pollutants, whose combined effects surpass our current understanding and preconceptions regarding toxicity and ecotoxicity.

This study highlights that these mixtures of pollutants can also have an indirect impact on our health by affecting the microorganisms present in the water. Such pollution could potentially impact the ability of pathogenic microorganisms to infect us. This concept has gained increasing interest, with studies focusing on humans, particularly on their various microbiota, such as the skin microbiota. The findings indicate that pollution mixtures can affect the virulence potential of certain waterborne pathogenic bacteria, even at concentrations currently observed in rivers. Further investigations could yield more significant results by examining different virulence genes for the two bacteria studied. Rivers, as stressful environments, have the potential to enhance the virulence of bacteria. While this risk may not be increased for certain genes of *S.* Typhimurium, it could amplify specific virulent factors of *P. aeruginosa* H103, such as motility, under the influence of a five-fold concentration of the pollution cocktail, as tested in this study. Importantly, this research underscores the interconnections between environmental health and public health security, emphasising the significance of considering the environment and its pollution.

AUTHOR CONTRIBUTIONS

J. L. and L. M. are the head of the project, are responsible for designing the project, and gave the initial idea. R. A. H. I. A. A., M. A. B., L. M., and J. L. focused on analytical chemistry. R. A. H. I. A. A., A. C., and J. M. B. worked on microbiology. J. L. and L. M. rendered support in database exploitation (French water agency). J. L., L. M., and R. A. H. I. A. A. wrote the original draft of the paper. J. L., L. M., R. A. H. I. A. A., J. J., J. M. B., A. C. wrote the review and edited the article.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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