# THE FRESH WATER MUSSEL *UNIO MANCUS* AS SENTINEL FOR ANTIBIOTIC-RESISTANT ENTEROBACTERIACEAE PRESENT IN INLAND WATERS

Master's Degree in Zoonoses and One Health

*Academic course 2018-2019*

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This thesis was part of the Master's Degree in *Zoonoses and One Health*, carried out at the *Universitat Autònoma de Barcelona* during the academic course 2018-2019. The final work is a product of join effort from the student Lourdes Lobato and their mentors: Dr. Óscar Cabezón, Dr. Lourdes Migura-Garcia and Dr. Xavier Fernández-Aguilar. Also, further acknowledgements are given in the next section.

Cover picture: *The Naiad*, John William Waterhouse (1893)

**ACKNOWLEDGMENTS**

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Last but not least, it has been a pleasure to learn laboratory techniques from Mrs. Marta Rebull and I really thanks her and my friend and colleague Irene Torres for the incalculable help at the laboratory.

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**LIST OF ABBREVIATIONS**

AMR: Antimicrobial resistance

CFU/mL: Colony-forming units per millimeter

ECDC: European Centre for Disease Prevention and Control

ECOFF: Epidemiological cut-off

EMA: European Medicines Agency

EU: European Union

EUCAST: European Committee in Antimicrobial Susceptibility Testing

FAO: Food and Agriculture Organization of the United Nations

IUCN: International Union for Conservation of Nature

LC: Location point

MDR: Multidrug resistance

MIC: Minimum Inhibitory Concentration

MS: Member States

NE-Spain: Northeast-Spain

OIE: World Organisation for Animal Health

PH: Public Health

PRAN: Plan Nacional frente a la Resistencia a los Antibióticos

WHO: World Health Organization

**Abstract**

Freshwater mussels are known to provide ecosystem services in many ways. Their natural filter-feeding activity contributes to maintenance of water sources and also, they are considered good bio-indicators of water contamination. *Unio mancus* is a threatened naiad inhabiting inland freshwaters from the Mediterranean basin, whose population has been decreasing mainly due to degradation of the habitat by human activities. In the present study, *U. mancus* was tested as a tool for detecting Enterobacteriaceae from inland freshwater. For that purpose, two experiments were carried out. The first one evaluated the capability of these naiads to filter and retain inoculated cephalosporin-resistant *E. coli* in laboratory conditions. They proved to maintain bacterial loads in their soft tissue up to 4 days post-exposure. The second experiment was carried out between July 2018 and April 2019 along *L'Estany de Banyoles* Lake and its scraping irrigations. To determine the presence of antimicrobial-resistant Enterobacteriaceae under different levels of anthropogenic pressure, underwater caged naiads were tested for five periods of 15 consecutive days. Among all isolated bacteria using *Vitek®2 system*, six MDR-*E. coli* and one MDR-*Klebsiella pneumoniae* were found in the most anthroponized and polluted location points. Minimum inhibitory concentrations (MIC) for these bacteria were interpreted according to the Epidemiological cut-off (ECOFF) values from the European Committee in Antimicrobial Susceptibility Testing (EUCAST). Our results suggest that *U. mancus* has potential to become sentinel of bacterial pathogens of Public Health concern. A better understanding of their filtration capability will provide insights into more accurate cost-benefit analyses for their repopulation in autochthonous freshwater systems.

## 1. Introduction

The connection between the health of animals, plants, people and shared environment is well recognized by One Health concept. Although the role of the environment is still less appreciated in Public Health (PH) research, it is well known that by integrating environmental factors in the One Health strategy, a better understanding of some PH concerns can be achieved. The study of the ecology of pathogens in the environment improves the identification of health risks before the potential exposure to the threat. In addition, it has long been recognized that wild animal populations can potentially act as sentinels for several zoonotic pathogens (Fox, 2001).

Antimicrobial resistance (AMR) is a natural selection mechanism in which a bacterial population becomes capable to multiply and/or survive under the presence of an antimicrobial agent in comparison with the wild type bacterial population of the same species. Intensive farming and human overcrowding have triggered serious environmental degradation and changes in water patterns. These factors, along with an inappropriate use of antimicrobials in human and veterinary medicine among others, have accelerated the emergence of resistant bacteria and nowadays it is considered a worldwide PH problem (Aslam *et al.*, 2018). In the last decades, the increasing numbers of multi-drug resistant Gram-negative bacteria causing infections in hospital settings has focused our concern on Enterobacteriaceae, which are part of human and animal gut microbiota (Walsh, 2018). Combating the emergence AMR is addressed by international institutions such as the *World Health Organization* (WHO), *Food and Agriculture Organization* of the United Nations (FAO) and the *World Organisation for Animal Health* (OIE) under a One Health approach (World Health Organization, 2016).

As a result, WHO has focused on developing a global action plan for tackling AMR, to which many Member States (MS) have joined. Furthermore, at European Union (EU) level and National level, coordinated actions are being implemented with a cross-sectional approach: PH, animal health and environmental health. The *European Food Safety Agency* (*EFSA*) works in conjunction with other EU organisms such as the *European Centre for Disease Prevention and Control* (*ECDC*) and the *European Medicines Agency* (*EMA*), gathering and analysing data from the MS regarding AMR and zoonoses. National control programmes of bacteria causing foodborne zoonoses are already implemented at national level, like for *Salmonella* in poultry production, which are being used to monitor AMR. The Decision 652/2013 also establish the screening and notification of antibiotic resistances in

zoonotic and commensal bacteria and other microorganisms such as *Campylobacter coli, Campylobacter jejuni, Escherichia coli*-indicator and extended-spectrum β-lactamases (ESBL)/AmpC β-lactamases producing *Escherichia coli* (*E. coli*) are also monitored (Ministerio de Agricultura Pesca y Alimentación, 2016). In Spain the "*Plan Nacional frente a la Resistencia a los Antibióticos (PRAN)*" is in charge of controlling the risk of selection and dissemination of AMR, and thus, diminishing their impact on PH and animal health by preserving the efficacy of the existing antibiotics. The PRAN issues a report called JIACRA-ES, which integrates the relationship between antibiotic consumption and resistance development. For example, data presented in this report demonstrates a clear correlation between increasing numbers of cefotaxime-resistant *E. coli* over the years and the use of 3$^{rd}$ and 4$^{th}$ generation cephalosporins in human medicine.

Five antibiotic groups are categorized as critically important for Public Health: carbapenems, 4$^{th}$ generation cephalosporins, (fluoro)quinolones, macrolides and polimixines. Except for carbapenems, all of them are also registered in veterinary medicine. For example, 3$^{th}$ and 4$^{th}$ generation cephalosporins are used in pig and bovine production, but they are also used in hospitals for the treatment of human infections caused by Gram-negative bacteria such as *E. coli* (PN Resitencia Antibióticos, 2016). *E. coli* often exhibits resistance to cephalosporins, fluoroquinolones and also can carry resistance genes for last-line macrolides such as azithromycin. To a great extent, the drug resistance increase in Enterobacteriaceae has been mainly due to an increase in ESBL, whose genes are generally located on plasmids, extrachromosomic DNA that can be transferred between different bacterial species (Hazen *et at*, 2017). Since Enterobacteriaceae are part of the gut microbiota, the main spread of ESBL genes to the environment comes from animal and human residues (Overdevest *et al.*, 2011).

Water sources are prone to get contaminated by natural events such as floods and additional side effects from urbanization. Continuous and substantial releases of sewage, chemical compounds, heavy metals and other contaminants are widely reported in urban streams and other inland freshwaters (Burket *et al.*, 2019; Ricciardi and Rasmussen, 1999). In addition, all of these processes can trigger the occurrence of diseases from waterborne pathogens. Water samples are fairly easy and simple to analyse but due to natural climate events such as rainfalls, flow rates and temperature flow, temporal sampling do not necessarily reflect contamination in watercourses and neither allow for spatial distribution analysis of pathogens (Palos Ladeiro *et al.*, 2015). Freshwater is specially subjected to quality analyses and many methods have been developed to provide safe drinking water. First steps

are mostly based on sedimentation and filtration treatments. However, limited consideration has been given to those natural mechanisms that already exist on aquatic ecosystems. These mechanisms provide free maintenance and decontamination of water sources, in addition to advocate for biodiversity and healthy ecosystems. An example of ecosystem services is the water filtration carried out by bivalve molluscs during the feeding process. These sedentary organisms reside in sediment and consume bacteria, phytoplankton, detritus and organic matter besides others, translocating nutrients along the water column and making resources available to other organisms helping to maintain the quality of the aquatic environment (Aceves *et al.*, 2018; Burket *et al.*, 2019).

*Unio mancus* (Phyllum Mollusca; Class Bivalvia) is a naiad inhabiting inland freshwaters from NE-Spain, France and Switzerland and throughout the Mediterranean region (Figure 1; see on *Annexes*). It is classified as a "near threatened" by the *International Union for Conservation of Nature* (*IUCN*) Red List and it is included in the National Catalogue of Endangered Species of Spain. Its population has been decreasing mainly because of the degradation of the habitat by residential and commercial development (urban areas, industry or tourism besides others), pollution and invasive species. Clean water free from pollution is an indispensable requirement for the naiads and its presence of this species has been reported in rivers, lakes and freshwater channels.

As a filter-feeding parasitic bivalve, the life cycle of *U. mancus* is complex and requires several development phases to achieve the maturity stage. One of the main requirements is the need to parasitize a fish to develop into a juvenile stage. Some of their specific host fishes known are *Squalius* spp., *Barbus haasi*, *Luciobarbus graellsii*, *Parachondrostoma miegii*, *Phoxinus phoxinus* and *Salaria fluviatilis* (Lopes-Lima, 2014). The life cycle starts in spring when an adult male (over 3 years-old) releases spermatozoids into the water and the adult female captures them and proceeds to an internal fertilisation (Figure 2). After this, thousands of larvae called *glochidiums* are released into the water. *Glochidiums* are parasitic larvae so they need to find a fish and settle into its gills, where they will develop into a small cyst and feed with the host's blood. After 7-20 days of development, *glochidia* evolves into a juvenile. Juveniles are then released back into the water where they fall onto the sediment and will start the stage of adultness until they reach the sexual maturity at the age of 3-4 years (LifeUNIO, 2019).

Survival rate of *U. mancus* is low throughout the pre-adult stages of the life cycle due to difficulties to find an appropriate host during larvae parasitic phase and the correct settle of the juveniles. The chances of *U. mancus* survival therefore rely on a big reproductively active population, good population structure of autochthonous fishes and the absence of diseases and pollution in the environment.

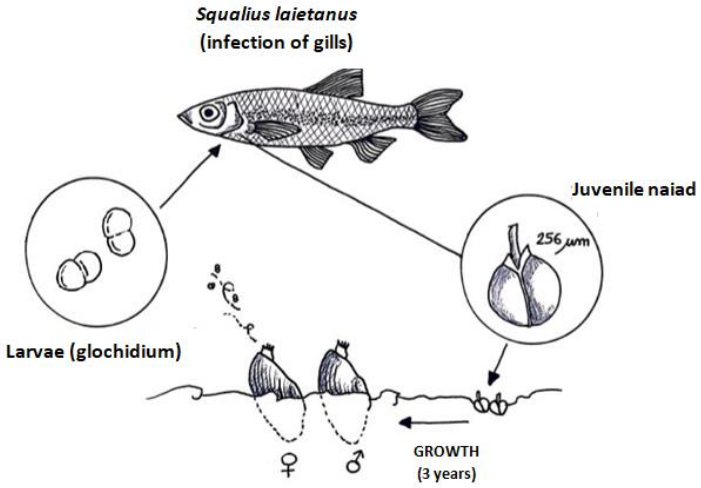



**Figure 2.** *Unio mancus* life cycle. *Squalius laietanus* is a freshwater fish autochthonous from Spain, Andorra and France and it is one of the main parasitized host by *U. mancus* in these regions.

Most of the literature on freshwater mussels have focused on bioaccumulation of contaminants and pathogens. Several filter-feeders such as zebra mussel (*Dreissena polymorpha*), Alabama rainbow mussel (*Villosa nebulosa*) and the Golden freshwater clam (*Corbicula fluminea*) are focus of study by scientist around the world. Previous studies have investigated the bioaccumulation and elimination kinetics of microorganisms by molluscs and have determined their vectorial capacity regarding bacterial, viral and protozoa pathogens such as *E. coli* and *Clostridium perfringens* (Burkhardt and Calci, 2000), Avian Influenza virus (Huyvaert *et al.*, 2012), Norwalk-like virus (Le Guyader *et al.*, 2006) and *Toxoplasma gondii* (Palos Ladeiro *et al.*, 2014; Palos Ladeiro *et al.*, 2015), besides others. *Dreissena polymorpha* is particularly considered a bivalve with a strong potential in reducing *E. coli* counts in freshwater systems (Mezzanotte *et al.*, 2016). Besides that, bivalves also have the ability to filter and accumulate pharmaceutical and other chemical compounds from aquatic ecosystems, and thus are considered good biological models in ecotoxicological studies. (Burket *et al.*, 2019).

Although *Unio mancus* may have the ability of preserving and restoring impaired mediterranean ecosystems as other bivalve molluscs already studied, there is a lack of research about the importance of this species and their potential role as a sentinel.

## 2. Objectives

The aim of this study is two-fold: first, to evaluate if *Unio mancus* is likely to become an appropriate sentinel for the monitoring of pathogens of Public Health concern as *E.coli* under a controlled environment in the laboratory. Second, to determine the presence of antimicrobial resistance in freshwater systems under different levels of anthropogenic pressure using the freshwater mussel *Unio mancus* as sentinel.

In addition to working on the objectives described above, it is expected to give a boost to the conservation of this species by recognizing its importance on the aquatic environment.

## 3. Materials and Methods

### 3.1 Experimental exposure

#### *Naiads collection and preparation*

Twenty-six juvenile (approximately 3 years old) *Unio mancus*, with a length of 20-30 mm, were collected from the breeding centre *Consorci de l'Estany* (Figure 3; see on *Annexes*) on 11$^{th}$ of April 2019 and transported alive to *Universitat Autònoma de Barcelona* for the experimental exposure study. They previously underwent a process of two days of fasting in a water tank without sediment, called depuration, in order to ensure that naiads purged any possible level of *E. coli* and other microorganisms.

The experimental use of captive-breeding naiads from *Consorci de l'Estany* was authorized by *Generalitat de Catalunya* government.

### *Experimental system setup*

The experiment was designed to ascertain if *U. mancus* was able to filter and retain *E. coli* loads and to assess the shedding time. Twenty-six naiads were used for this purpose.

Laboratory room temperature was set at 18ºC with air conditioning to avoid temperature oscillation and to ensure that naiads underwent no thermal stress. Water pumped from *L'Estany de Banyoles* was brought to the laboratory and placed in 25L plastic tanks (tapperware-like containers). To continually monitor the temperature in the water tank, a submersible thermometer was used. Filtered and autoclaved silica sand about 0.5-1 mm was used as a sediment, which was placed in a semi-floating polyvinyl chloride (PVC) cylinder with a pinpoint grid at the bottom. A submersible water pump (*Syncra Nano Multifunction Pump, SICCE*®) was attached to the tank wall to achieve uniform mixing and constant recirculation of water (Figure 4 and 5).



**Figure 4.** General setup of the experiment. See water tank with PVC cylinder holding inside, with a water pump connected and naiads waiting for the inoculation process in plastic boxes. **Figure 5.** Closed details of the experiment. PVC cylinder with silica sand sediment at the bottom and settled naiads. The PVC cylinder is holding from the plastic tank. Because it is semi-floating and has a grid bottom, water can recirculate from the outer to the inner part of the cylinder thanks to the water pump.

### *Inoculum preparation*

For the inoculum, an *E. coli* with a known mechanism of resistance to cephalosporins was selected (*E. coli* CTX-M-14). Filtration capacity of juvenile naiads in one hour was estimated at 40mL (Ostrovsky, Gophen and Kalikhman, 1993). A concentration of $1.5 \cdot 10^5$ CFUs/mL was prepared as a safe inoculum, after performing a literature review about experimental infection with bivalve species (Gu and Mitchell, 2002). Knowing the final

volume for the inoculation procedure (40mL) and starting with an *E. coli* concentration of $1.5 \cdot 10^8$ CFUs/mL (0.5 McFarland turbidity), the inoculum volume needed was calculated with the next formula:

$$[\,]_i \cdot V_i = [\,]_f \cdot V_f$$

where $[\,]_i$ was the initial concentration of *E. coli* used for preparing the solution; $V_i$ was the unknown initial volum of the inoculum; $[\,]_f$ was the chosen *E. coli* concentration for the inoculation; and $V_f$ was the final volume filtered by naiads in one hour. The calculated initial volume of the inoculum ( $V_i$ ) was 40µL.

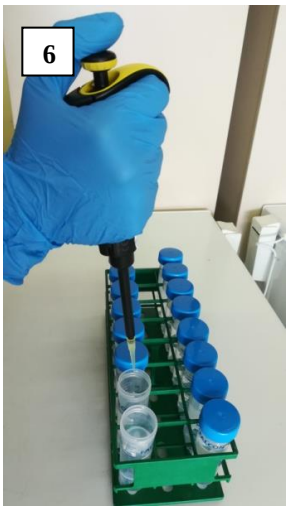




**Figure 6.** Individual pipette of 40µL of inoculum in Falcon™ tubes containing one naiad each one. **Figure 7.** Detail of the opened naiad's syphon (*), used as a filtering verification.

Once the final volume of *E. coli* was calculated, the 26 naiads were placed with the siphon in upright position in 50mL Falcon™ tubes independently with 40mL of bottled water. 40µL of *E. coli* inoculum were pipetted in 27 Falcon™ tubes with naiads and were left to rest in a quiet surface (Figures 6 and 7). The two remaining naiads were used as negative control samples and no inoculum was used.

***Sample collection***

Sample collection was organized in subsets of three naiads for each time point, selecting roughly one naiad of each small, medium and large size. Also, a 1L tank water sample was collected for every naiad's sampling for further analyses.

After one-hour exposure to the inoculum, naiads were rinsed with water from *L'Estany de Banyoles* and were placed in the silica sand sediment of the water tank, except for the two control naiads and three inoculated naiads, which were directly brought to the laboratory for the first analyses. Along with these five first naiads, a microalgae feed sample and 1L of tank water were also taken for analyses. Eight hours post-inoculation three more naiads were collected and brought to the laboratory to analyse. Sampling times were then defined every 24 hours for eight days, following a strict protocol of tank disinfection and maintenance.

***Water tank maintenance and feeding***

After every sample collection, the dirty tank was emptied, washed up and correctly disinfected with a solution of sodium hypochlorite. Approximately one cup of commercial 2% sodium hypochlorite solution was used in a 25L water tank, obtaining a 0.05% sodium hypochlorite solution. Later on, the tank was carefully rinsed and let dry for the next use. Silica sand was rinsed and autoclaved as well. Two identical tanks were needed to move naiads from one to another during cleaning and disinfection procedures. Just before the tank swap, naiads were rinsed with clean water from *L'Estany de Banyoles* and placed in upright position on the clean sediment. Thereafter, the remaining naiads in the tank were fed *ad libitum* with a commercial formula of lyophilised microalgae (*Easy Reef, Blueclownfish* Company).

***Tissue and water analyses***

Once in the laboratory, naiads were weighted, measured and recorded individually. Subsequently, they were dissected and processed as follows. The whole organism (soft tissue) was ground individually with 1mL phosphate-buffered saline solution (PBS) and homogenized using a vortex. From each of the resulting suspensions, 100µL were pipetted in

the middle of two different mediums: McConkey and McConkey agar supplemented with ceftriaxone (2mg/L); and streaked with a spreader (*Digralski* handle). Streaked plates were brought to incubation at 37ºC for 24 hours and naiad's suspensions were kept at -80ºC.

One litter water samples were filtered with a paper filter (*Durapore*® membrane filters 0.45µL) (Figure 8; see *Annexes*). Filters were later homogenized with 10mL PBS and vortexed. From this suspension, 100µL were pipetted on same agar media described above and streaked with a spreader (*Digralski* handle). Streaked plates were brought to incubation at 37ºC for 24 hours and filter's suspensions were stored at -80ºC.

### 3.2 Sentinel study

#### *Study location*

This field study was carried out in Girona Province, Catalonia, from *L'Estany de Banyoles* lake and along its scrapping irrigations: *Canaleta* stream and *Terri* River. *L'Estany de Banyoles* is located in *Banyoles* city and it is the largest semi-natural lake in Catalonia, with a length of more than 2,000 meters and covering an area of approximately 112 ha (Figure 9 and 10).

The naiads used as sentinels came from the breeding centre *Consorci de l'Estany*, supported by *LIFE Projecte Estany [LIFE08 NAT/E/000067]*, which ultimate goal is to help *Unio mancus* conservation by improving the knowledge of the naiad's reproductive biology, stablishing a strong breeding in captivity and reintroduction programme of naiads and becoming a reference centre at a National and European level.

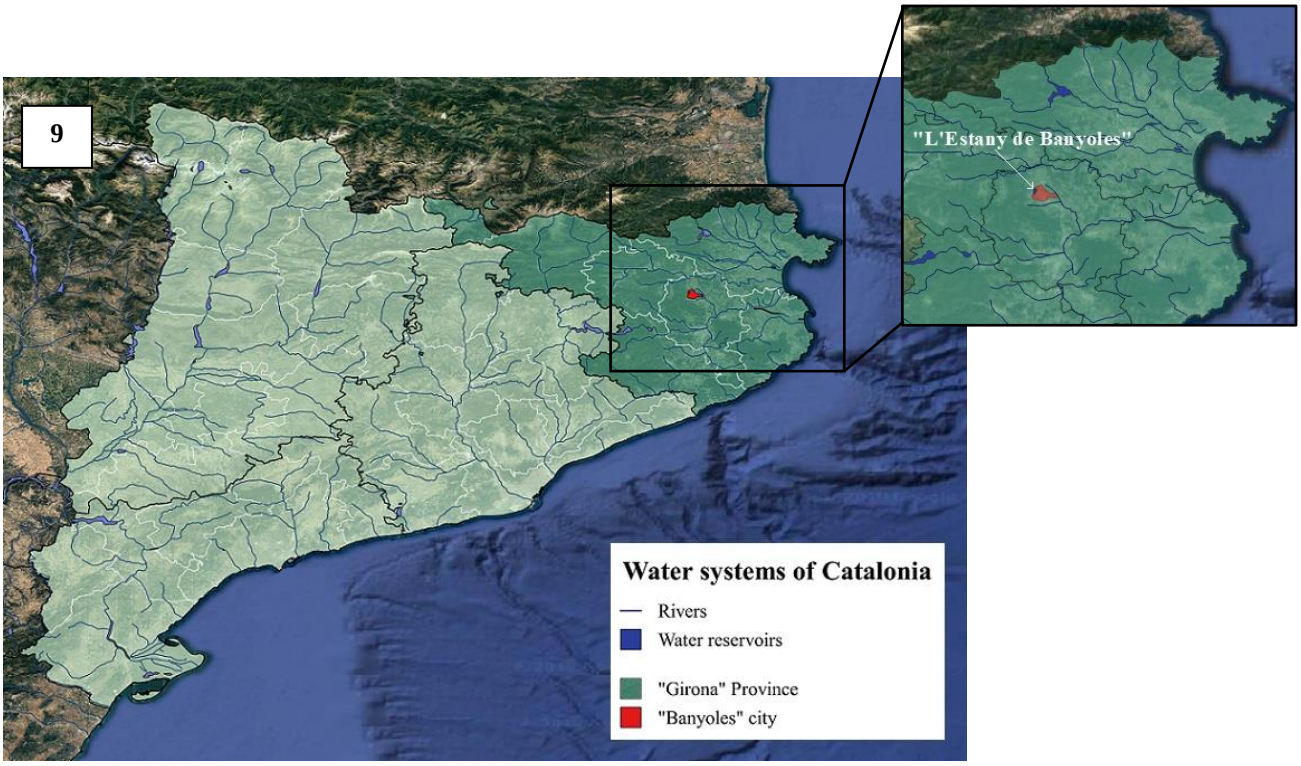



**Figure 9**. Location of the study and water systems of Catalonia (*QGIS*).

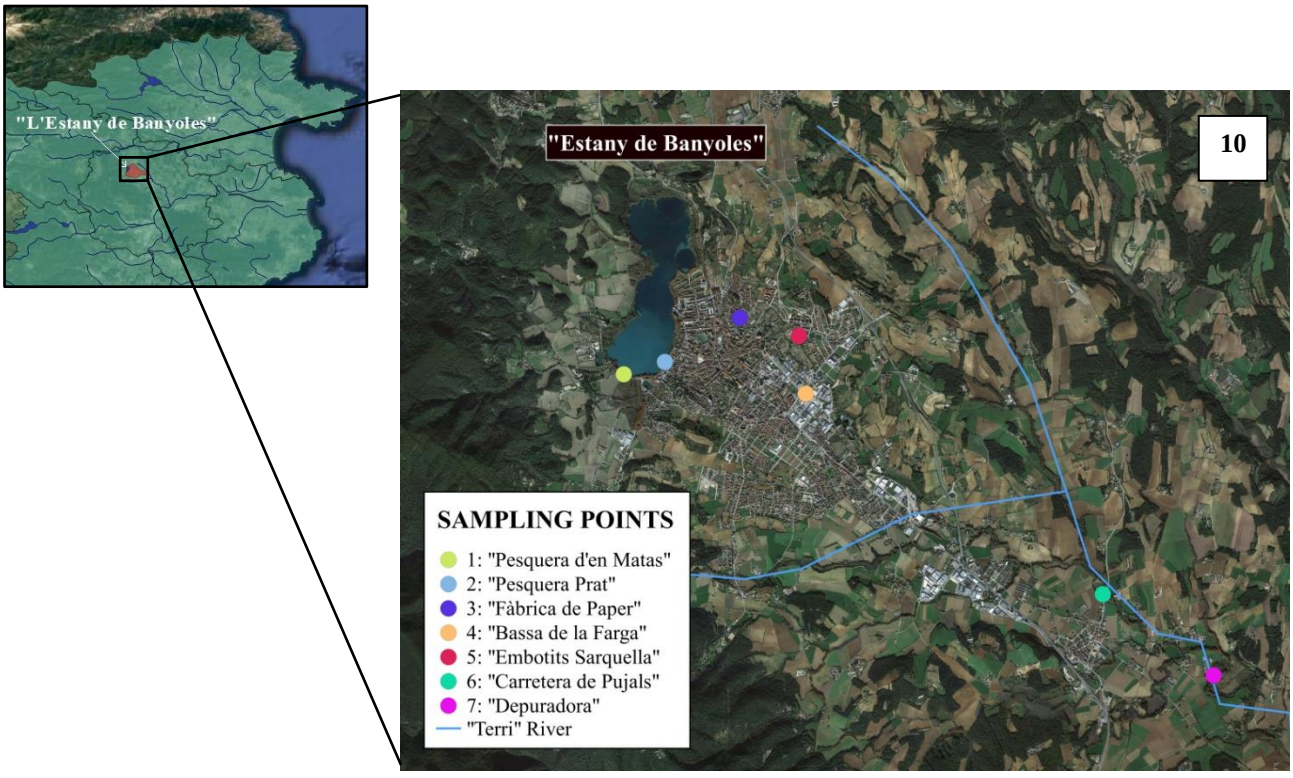



**Figure 10.** Sampling points location in *Banyoles* city. Points 1 and 2 are located in the lake; from point 3 to 5, samples are located throughout *Banyoles* city following *L'Estany de Banyoles* scrapping irrigations, mainly *Canaleta* stream (not showed in the map). Points 6 and 7 are located along the *Terri* River (*QGIS*).

***Field exposure and sample collection***

From July 2018 to April 2019, five exposure periods on the field were organized, with a length of two weeks each one and separated each other by approximately one month. For every exposure period, twelve juvenile naiads (*Unio mancus*) from the breeding centre *Consorci de l'Estany* were placed in underwater boxes from point 1 to point 7 respectively. After exposure time, two naiads from each cage were collected and transported inside 50mL Falcon™ tubes to the *Universitat Autònoma de Barcelona* for analyses. The remaining naiads in underwater boxes were used for repopulating the river basins of the area, under the supervision of the breeding centre *Consorci de l'Estany*.

***Sample purification and tissue analysis***

Once in the laboratory, naiads were dissected avoiding cross-contamination and immediately after, whole organisms were individually ground with 1 mL phosphate-buffered saline solution (PBS) and vortexed to resuspend the pellet (Figure 11 and 12).

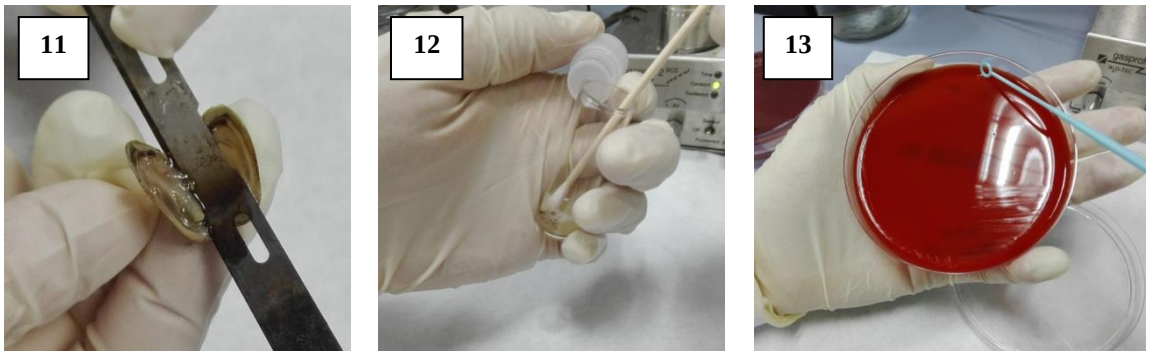



**Figure 11**. Sterile dissection of a naiad. **Figure 12**. Grounding of naiad's tissues (whole organism) with 1 mL PBS. **Figure 13**. A three quadrant-streaking in Agar medium plate with naiad's resuspension.

For the detection of Gram-negative and enteric bacteria, a tissue resuspension from each individual was used to perform three quadrant-streakings of Petri dishes containing respectively blood agar (Figure 13), McConkey and McConkey agar supplemented with ceftriaxone (2mg/mL). Plates were incubated at 37ºC for 24 hours. Following incubation, isolated colonies were re-streaked on the same agar-medium and were incubated for 24 hours to obtain a pure culture. Finally, isolated colonies were ground individually with 1 mL of a

solution containing 80% of brain-heart infusion (BHI) and 20% of glycerol. These suspensions were stored at -80ºC for later identification.

For *Salmonella* detection, 100µl of each naiad's tissue suspension were mixed with 900µl of buffered peptone water (BPW) and were incubated for 24 hours at 42ºC. After the incubation period, 100µl of this BPW suspension were inoculated in a semi-solid Rappaport-Vassiliadis medium (RV) and incubated for 24 hours at 42ºC. Results from the RV incubation was observed at 24 hours and if no colour change occurred, 24 more hours of incubation was implemented. RV plates with no colour change (staying malachite green) at 48 hours were classified as negative for *Salmonella* growing, whereas those plates turned white were classified as positive and a later re-streaking on XLT4-agar medium was performed. Black or black centred colonies from the XLT4-agar were ground with 1 mL of a solution containing 80% BHI and 20% of glycerol, and stored at -80ºC for a future typification.

***Microbiological identification***

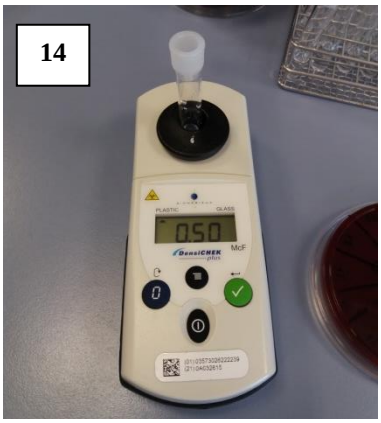
All bacterial isolates were tested on *VITEK®2 system* with Gram-negative identification cards. Suspensions were prepared by emulsifying bacterial isolates, previously stored at -80ºC and re-streaked in blood agar medium 24 hours before, in 0.45% sodium chloride solution and a 0.6 McFarland turbidity standard was adjusted for each one. Suspensions and Gram-negative identification cards were loaded into the VITEK®2 system according to the manufacturer's recommendations and results were automatically reported by its software in less than 24 hours.

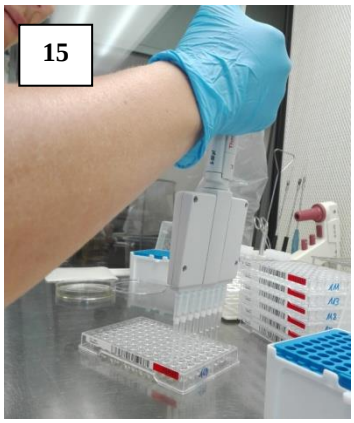
***Minimum Inhibitory Concentration (MIC)***

To determine MICs of relevant isolates, inoculums with a turbidity of 0.5 McFarland in a 0.85% sodium chloride solution were prepared (Figure 14). For that purpose it was necessary to previously re-streak these bacterial isolates in blood agar and incubate them for 24 hours. Once 0.5 McFarland solutions were prepared, 10 µl were inoculated in 10 mL of Mueller-Hinton Broth (MHB) respectively. For the control, the *E. coli* ATCC -25922 was also prepared using the same procedure. Afterward, using a multichannel pipette, 50 µl of each inoculated MHB solution was placed in *Thermo Scientific™ Sensititre™ Gram-negative MIC* plate (Figure 15) and brought to incubation at 37ºC for 24 hours. Antimicrobials tested were:

gentamicin (0.5-32µg/mL), ampicillin (1-64µg/mL), cefotaxime (0.25-4µg/mL), ceftazidime (0.5-8µg/mL), meropenem (0.03-16µg/mL), chloramphenicol (8-128µg/mL), azithromycin (2-64µg/mL), ciprofloxacin (0.015-8µg/mL), nalidixic acid (4-128µg/mL), sulfamethoxazole (8-1024µg/mL), trimethoprim (0.25-32 µg/mL), tetracycline (2-64 µg/mL), tigecycline (0.25-8µg/mL) and colistin (1-16µg/mL).

Reading of the plates was performed after the incubation period, using a mirror (Figure 16). Epidemiological cut-off (ECOFF) values were interpreted according to the European Committee in Antimicrobial Susceptibility Testing (EUCAST). In this study, the term "resistant" referred to presence of acquired and mutational mechanisms of resistance in bacteria, not from a clinical but from an epidemiological point of view. In that respect, "non-wildtype" term was replaced on the results by "resistant".



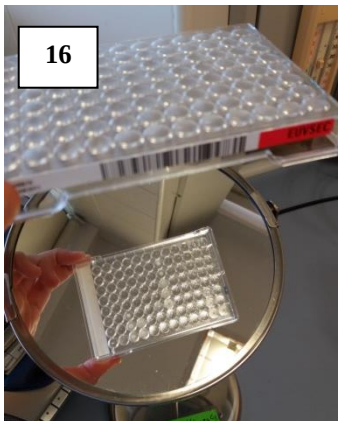




**Figure 14.** Adjusted 0.5 McFarland turbidity. **Figure 15.** Multichannel pipetting in *Thermo Scientific*™ *Sensititre*™ *Gram-negative MIC* plates. **Figure 16.** Plate reading using a mirror.

## 4. Results

### 4.1 Experimental exposure

#### *U. mancus can reduce ceftriaxone-resistant E. coli loads from freshwater in the laboratory*

An initial concentration of $1.5 \cdot 10^5$ CFUs/mL of ceftriaxone-resistant *E. coli* was inoculated on naiads. Negative control samples were two naiads who were not inoculated and a water sample from the tank and algae for feed were also analysed. All four negative control samples showed no bacterial growth.

Bacterial load on naiad's tissue was detected up to the 4th day of sampling (96 hours post-inoculation) and water samples from the tank were positive until the 3rd experimental day. Two consecutive negative samplings were obtained after the 4th day.

For every sampling time, no correlation was observed between soft body weight and CFU/mL detected on naiad's tissue. However, all naiads with the smaller soft body weight retained a maximum of $1.65 \cdot 10^2$ UFC/mL (in the first sampling time), whereas medium sized and large naiads achieved maximum *E. coli* counts about $2.46 \cdot 10^3$ and $2.32 \cdot 10^3$ UFC/mL respectively in the second sampling time (Table 1 and Figure 17).

**Table 1.** Calculated ceftriaxone-resistant *E. coli* UFC/mL found along the experiment. Results are classified by sampling times, where T1=1h post-exposure; T2=8h post-exposure; T3=24h post-exposure (1 day); T4=48h post-exposure (2 days); T5=72h post-exposure (3 days); T6=96h post-exposure (4 days); T7=120h post-exposure (5 days); T8=144h post-exposure (6 days). [1]Naiad's size visually classified as small before soft body weight calculated. [2]Naiad's size visually classified as medium before soft body weight calculated. [3]Naiad's size visually classified as large before soft body weight calculated. Overall, no correlation is observed between soft body size and CFU/mL calculated per sampling time.

| | | **SAMPLING TIME** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **T1** | **T2** | **T3*** | **T4** | **T5** | **T6** | **T7** | **T8** |
| **BW[1] 1** | **Soft body weight (g)** | 0.2 | 0.1 | 0.3 | 0.3 | 0.2 | 0.2 | 0.4 | 0.3 |
| | ***E.coli* CFU/mL** | $1.65 \cdot 10^2$ | 25 | 15 | 15 | 0 | 15 | 0 | 0 |
| **BW[2] 2** | **Soft body weight (g)** | 0.5 | 0.3 | 0.4 | 0.5 | 0.6 | 0.5 | 0.7 | 0.5 |
| | ***E.coli* CFU/mL** | $1.2 \cdot 10^3$ | $2.46 \cdot 10^3$ | 10 | 55 | 5 | 5 | 0 | 0 |
| **BW[3] 3** | **Soft body weight (g)** | 0.9 | 0.9 | 0.7 | 1.1 | 0.9 | 0.8 | 0.7 | 0.7 |
| | ***E.coli* CFU/mL** | $2.6 \cdot 10^2$ | $2.32 \cdot 10^3$ | 5 | 10 | 0 | 10 | 0 | 0 |

The mean for the whole body weight of total naiads was about 2.3g, the shell weight was about 1.2g and the length was about 26.5mm. The mean for the soft body weight was 0.5g, although individual values can be seen on Table 1.

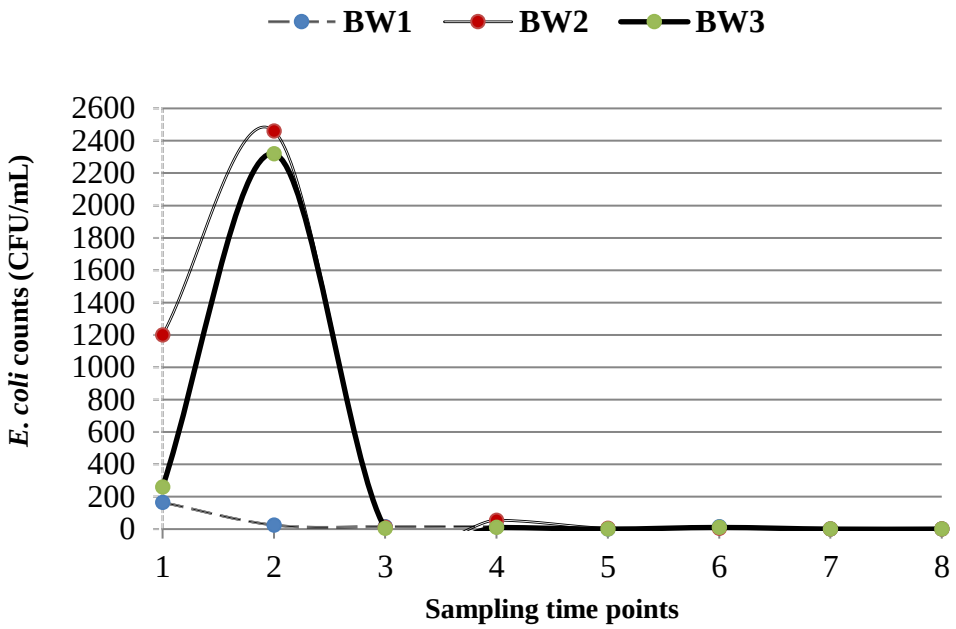



**Figure 17**. Evolution on *E. coli* counts (CFU/mL) on naiad's tissue along time (sampling time points) and classified by body weight (BW1=smallest; BW2=intermedium; BW3=largest).

## 4.2 Sentinel exposure

### *Capability of U. mancus to filter and retain Enterobacteriaceae from freshwaters systems*

The preliminary study revealed the capability of *U. mancus* to filter and retain inoculated *E. coli* in freshwater under a controlled environment in the laboratory. To verify this ability on the field, naiads from the breeding centre *Consorci de l'Estany* were tested along the *L'Estany de Banyoles* lake and its scrapping irrigations (*Canaleta* stream and *Terri* River) from July 2018 to April 2019. Collectively, eleven different *genera* of bacteria were identified with *VITEK®2 system*. All different microorganisms identified, listed in alphabetical order, were the next: *Aeromonas hydrophila/caviae* (low discrimination obtained by *VITEK®2 system*), *A. sobria*, *A. veronii* (Figure 18; see on *Annexes*), *Citrobacter braakii*, *C. freundii*, *E. coli* (Figure 18 and 19; see on *Annexes*), *Enterobacter cloacae*, *Klebsiella oxytoca*, *K. pneumoniae* (Figure 19; see on *Annexes*), *Leclercia adecarboxylata*, *Pantoea*

spp., *Pseudomonas mendocina, P. stutzeri, Raoultella planticola, Salmonella* spp., *Serratia plymuthica* and *Vibrio fluvialis*.

*E. coli* dominated all samples analysed, represented by 25.3% of the total sampled, followed by *Klebsiella* spp. with 23.9% (*K. oxytoca* 12.67% and *K. pneumoniae* 11.27%). Although specific procedures were executed to culture *Salmonella* spp., only one sample was found positive. Punctual isolation of *Serratia plymuthica*, *Pantoea* spp., *L. adecarboxylata* and *E. colacae* were found along all exposure periods. Two different *Pseudomona* genus were identified: *P. mendocina* (three positive samples during the same exposure time in two different locations) and *P. stutzeri* (punctual isolation). Two samples resulted positive for *V. fluvialis* during the same exposure time but different location point (close to charcuterie *Sarquella* and close to the water treatment plant). *R. planticola* was isolated during the third and fourth exposure periods, between November and late January.

***Effect of meteorological phenomena on bacterial isolates in sentinel naiads***

From late September 2018 to early February 2019 (three consecutive study-periods), bacterial growing and diversity was significantly greater than during any other time of the year (Table 2). Rainfall was identified as the determining factor of larger quantity and diversity of bacterial isolates. On November 15$^{th}$ 2019 a strong precipitation of 37 mm was registered in *Banyoles* city, where direct damage to river basins occurred, causing floods and strong water turbulences along *L'Estany de Banyoles* scraping irrigations. During this period, 11 different bacterial isolates were identified.

**Table 2.** Distribution of identified bacteria (diversity and abundance) obtained by *VITEK®2 system* classified by exposure time. Precipitation details are also indicated. [1]Microorganisms obtained from different colony morphologies growing on the plate.

| DATE | | MICROORGANISMS[1] (number) | TOTAL | RAINFALL |
|---|---|---|---|---|
| **Exposition** | **Collection** | | | |
| 20.07.2018 | 06.08.2019 | *A. veronii* (1)<br>*E. coli* (4)<br>*K. pneumoniae* (4)<br>*K. oxytoca* (1) | 10 | .<br>5 days of rain<br>(maximum 6.5 mm) |
| 21.09.2018 | 08.10.2018 | *A. hydrophila/caviae* (2)<br>*A. sobria* (1)<br>*C. freundii* (2)<br>*E. coli* (6)<br>*K. pneumoniae* (2)<br>*Salmonella spp.* (1)<br>*S. plymuthica* (1) | 15 | 6 days of rain<br>(maximum 11 mm) |
| 09.11.2018 | 26.11.2018 | *A. hydrophila/caviae* (2)<br>*A. sobria* (1)<br>*C. braakii* (1)<br>*C. freundii* (2)<br>*E. coli* (5)<br>*K. oxytoca* (6)<br>*K. pneumoniae* (1)<br>*L. adecarboxylata* (1)<br>*Pantoea spp*. (1)<br>*P. mendocina* (3)<br>*R. planticola* (1) | 24 | 10 days of rain, concentrated by the middle of the exposure time.<br><br>On Nov. 15$^{th}$, 37 mm of rainfall and floods were registered. |
| 25.01.2019 | 11.02.2019 | *A. hydrophila/caviae* (1)<br>*A. sobria* (4)<br>*C. braakii* (1)<br>*E. coli* (2)<br>*P. stutzeri* (1)<br>*R. planticola* (3)<br>*V. fluvialis* (2) | 14 | 8 days of rain<br>(maximum 3 mm) |
| 22.03.2019 | 08.04.2019 | *C. braakii* (1)<br>*C. freundii* (1)<br>*E. cloacae* (1)<br>*E. coli* (1)<br>*K. oxytoca* (2)<br>*K. pneumoniae* (1)<br>*S. plymuthica* (1) | 8 | 7 days of rain<br>(maximum 11 mm) |

***Effect of anthropogenic pressure on the nature of bacterial isolates in sentinel naiads***

To assess the effect of different levels of anthropogenic pressure (AP) on the diversity and abundance of bacterial isolates, results from all exposure periods were classified by location point (LP). Ceftriaxone-resistant strains were also indicated to give a better understanding of the nature of isolates. Location points were classified according to the following: A. Low anthropogenic pressure (fairly natural environment with an insignificant contact with human activities); B. Medium anthropogenic pressure (in close contact with human settlement and activity); C. High anthropogenic pressure (water collection of polluted irrigations from the city and/or proximity to an animal food industry). Results are reflected on Table 3.

LP with the strongest anthropogenic pressure showed the highest amount of bacterial isolates and the highest diversity of bacterial species. Furthermore, the presence of ceftriaxone-resistant strains was observed in all of these three locations, whereas resistant strains were not identified on locations with lower anthropogenic pressure.

A total of 24 bacterial isolates, from 11 different species, were obtained from samples coming from the water treatment plant (LP 7). Seven out of twenty-four isolates (30%) exhibited resistance to ceftriaxone. From charcuterie *Sarquella*, eight different bacterial species were obtained during the whole study period, with a total of 13 isolates. Seven of these bacterial isolates (54% of the total sampling) resulted resistant to ceftriaxone. Additionally, 15% of the 13 total isolates from *Pujals* Road also exhibited resistance to ceftriaxone. Both samplings from the lake showed scarce amount and diversity of bacterial isolates, reflecting minimal water pollution.

**Table 3.** Distribution of identified microorganisms using *VITEK®2 system* by location point. [1]Locations points correspond to the following: 1. *Pesquera d'en Matas* (Fishing industry *Matas*); 2. *Pesquera Prat* (Fishing industry *Prat*), 3.*Fàbrica de Paper* (Paper factory), 4. *Bassa de la Farga* (Dam *Farga*), 5. *Embotits Sarquella* (Charcuterie *Sarquella*), 6. *Carretera de Pujals* (*Pujals* Road) and 7. *Depuradora* (Water treatment plant). [2]For the anthropogenic pressure classification, environmental and human factors have not been considered (see previous explanation).

| | | **IDENTIFIED MICROORGANISMS** | | |
|---|---|---|---|---|
| **Location Point[1]** | **Anthropogenic Pressure[2]** | **Susceptible (number)** | **Resistant to ceftriaxone (number)** | **TOTAL (wildtype; resistant)** |
| **1** | **A** | *A. veronii* (1)<br>*R. planticola* (1) | | **2** |
| **2** | **B** | *C. freundii* (1)<br>*K. pneumoniae* (1)<br>*S. plymuthica* (1) | | **3** |
| **3** | **B** | *C. braakii* (1)<br>*E. coli* (2)<br>*K. oxytoca* (1)<br>*K. pneumoniae* (1)<br>*Pantoea* spp. (1) | | **6** |
| **4** | **B** | *A. hydrophila/caviae* (1)<br>*K. oxytoca* (3)<br>*K. pneumoniae* (2)<br>*L. adecarboxylata* (1)<br>*R. planticola* (1) | | **8** |
| **5** | **C** | *C. freundii* (1)<br>*E. coli* (2)<br>*K. oxytoca* (1)<br>*K. pneumoniae* (1)<br>*Salmonella* spp. (1) | *A. hydrophila/caviae* (2)<br>*A. sobria* (1)<br>*E. coli* (2)<br>*K. oxytoca* (1)<br>*V. fluvialis* (1) | **13**<br>(**6; 7**) |
| **6** | **C** | *A. hydrophila/caviae* (1)<br>*A. sobria* (3)<br>*C. braakii* (1)<br>*C. freundii* (1)<br>*E. cloacae* (1)<br>*E. coli* (4) | *A. hydrophla/caviae* (1)<br>*P. mendocina* (1) | **13**<br>**(11; 2)** |
| **7** | **C** | *C. braakii* (1)<br>*C. freundii* (2)<br>*E. coli* (5)<br>*K. oxytoca* (3)<br>*K. pneumoniae* (3)<br>*R. planticola* (2)<br>*S. plymuthica* (1) | *A. sobria* (1)<br>*E. coli* (2)<br>*P. mendocina* (2)<br>*P. stutzeri* (1)<br>*V. fluvialis* (1) | **24**<br>**(17; 7)** |

### *Minimum inhibitory concentrations*

From all identified bacteria obtained by *VITEK®2 system*, 54 were selected to study their respective MICs. A total of 14 different species were analysed (Table 4).

However, conclusive results were obtained for six bacterial species (Tables 5-10; see on *Annexes*), since ECOFF values have not been described for the rest of the species of the present dataset.

**Table 4**. Isolated bacteria (n=50) obtained by *VITEK®2 system* and tested for MIC with *Thermo Scientific™ Sensititre™ Gram-negative MIC*. *Bacterial species with ECOFF values available on EUCAST.

| STRAIN | TESTED SAMPLES | STRAIN | TESTED SAMPLES |
|---|---|---|---|
| *Citrobacter braakii* | 3 | *Pseudomonas mendocina* | 1 |
| **Citrobacter freundii* | 6 | *Pseudomonas stutzeri* | 1 |
| **Enterobacter cloacae* | 1 | *Raoultella planticola* | 1 |
| **Escherichia coli* | 18 | **Salmonella sp.* | 1 |
| **Klebsiella oxytoca* | 9 | *Serratia plymuthica* | 1 |
| **Klebsiella pneumoniae* | 8 | *Vibrio fluvialis* | 2 |
| *Leclercia adecarboxylata* | 1 | *E. coli* ACTT | 1 |
| *Pantoea spp.* | 1 | | |
| **TOTAL TESTED SAMPLES**: 54 | | | |

Four resistant strains of *C. freundii* were found for ampicillin, whereas one was for cefotaxime and ceftazidime antimicrobials respectively (Table 6; see on *Annexes*). The only *E. cloacae* tested was pansusceptible (Table 6; see on *Annexes*). *E. coli* resistance was detected for azithromycin (n=6), followed by ciprofloxacin (n=5), cefotaxime (n=5), nalidix acid (n=5) and trimethoprim (n=5) (Table 7; see on *Annexes*). Six *K. oxytoca* strains from nine tested exhibited resistance to ampicillin and only one to ciprofloxacin (Table 7; see on *Annexes***)**. *K. pneumoniae* resistance was detected for ampicillin (n=6), cefotaxime (n=1), ceftazidime (n=1), ciprofloxacin (n=1) and tetracycline (n=1) (Table 9; see on *Annexes*). The *Salmonella* spp. showed phenotypic resistance to ampicillin (Table 10; see on *Annexes*).

From the 18 *E. coli* tested, 10 were resistant to at least one antimicrobial and 5 were multi-drug resistant (MDR): resistant to three or more antimicrobial. One *K. pneumoniae* was also MDR, exhibiting resistance to ampicillin, ceftazidime, cefotaxime, and ciprofloxacin.

Of the total isolates, the AMR to ampicillin was the highest (40%) amongst the tested antibiotics, followed by ciprofloxacin (12.3%) and cefotaxime (10.8%). All isolates were susceptible to azithromizin, tigecycline and colistin.

## 5. Discussion and conclusions

The present study assesses the capability of *Unio mancus* to act as a sentinel of pathogens of Public Health (PH) concern; in particular antimicrobial-resistant Enterobacteriaceae. Overall, results revealed that naiads were able to filter and retain bacterial loads under experimental and natural conditions and thus, confirm that naiads can be used as indicators of anthropogenic AMR contamination in freshwater systems.

Isolated bacteria from the *L'Estany de Banyoles* and its scraping irrigations were mainly *E. coli* and *Klebsiella* spp., although other genera were present in smaller number of samples: *C. braakii* and *C. freundii, P. mendocina* and *P. stutzeri, Raoultella planticola*, *Salmonella* spp., *Serratia plymuthica* and *Vibrio fluvialis.* These Enterobacteriaceae species are widely distributed in the environment (soil and water) and can be found in the intestinal microbiota of animals and humans. They often appear as opportunistic pathogens causing nosocomial infections, such as skin secondary infections (surgical wounds), urinary and intestinal tract infections, septicaemia and meningitis among others, but they are more representative of immune-compromised and elderly patients with underlying diseases (Atıcı *et al.*, 2018; Bisharat, 2012; Del Rosario Aragone *et al.*, 1992; Gani *et al.*, 2019; Manganello *et al.*, 2001; Mehmood *et al.*, 2018).

*Vibrio fluvialis* -reported in a wide range of coastal environments, seas, estuaries and brackish waters- is considered an emerging pathogen involved in diarrheal outbreaks mainly due to water and/or seafood consumption. Moreover, *V. fluvialis* appears to exhibit AMR phenotype more frequently than other *Vibrio* species. In the Mediterranean basin, *V. fluvialis* resistant to several antimicrobials have been found on fish farms (Ramamurthy *et al.*, 2014). Although *V. fluvialis* is currently considered an important emerging pathogen, Epidemiological cut-off (ECOFF) values for antimicrobial resistance are not available on EUCAST for this species. Efforts to study this pathogen should be made to rapidly identify resistance phenotypes and implement control strategies.

Six MDR-*E. coli* and one MDR-*K. pneumoniae* were identified by MIC determination. Particularly, *K. pneumoniae* is often the cause of assisted-ventilation intra-hospital infection and tends to display MDR (Cella *et al.*, 2017; Ostria-Hernandez *et al.*, 2018). On the other hand, *E. coli* is a waterborne pollutant generally associated with the waste and it is usually investigated as indicator of water quality. Also, *E. coli* often causes nosocomial infections (Oteo, J. and Lázaro, E., 2005). Additionally, from all isolated MDR-*E. coli* in this study,

80% were resistant to 3rd generation cephalosporins, quinolones and sulfonamides; and all of them resulted resistant to ampicillin. These results match research findings starting in the early 2000, with increasing numbers of resistance to fluoroquinolones and 3rd generation cephalosporins in *E. coli* -and *K. pneumoniae* (Eckert *et al.*, 2004)- along hospital settings and community-associated outbreaks (Oteo *et al.*, 2006).

In the present field study, all MDR bacteria were isolated from locations that can be considered with a higher anthropogenic influence, such as being near to meat industry or under the drainage of a water treatment plant. Animal food production has demonstrated to play an important role in the emergence of resistant bacteria, having a positive and direct association with the use of antimicrobials in farms (Chantziaras *et al.*, 2014; Österberg *et al.*, 2016). Resistant bacteria can be transmitted by several routes, including direct contact with animals, along the food-production chain and by contamination of water and soil surfaces with waste (Marshall and Levy, 2011).

In addition to the positive correlation between the level of anthropogenic pressure and the nature of the bacterial isolates, weather appears to influence as well. Throughout 15th November 2018, 37mm of precipitation fell over *Banyoles* city, increasing the turbidity and suspended solids in water, providing a likely higher organic substrate to naiads. Although the present study did not contemplate specifically this factor, heavy rainfall resulted in an increase in the abundance of isolated bacteria.

Bivalve shellfish, especially those for human consumption, haven been long investigated for the bacterial and chemical accumulation and elimination processes. These processes differ slightly between bivalve species, but a common mechanism to capture suspended particles in water and digest and/or eliminate them have been proposed (Perkins *et al.*, 2016). Several researchers have claimed that factors such as body size -weight and length- (Reeders and Bij de Vaate, 1990), algae concentration in water (Riisgård, Egede and Barreiro Saavedra, 2011) and water temperature (Pestana *et al.*, 2009) may interfere with filtering rates and even filtering saturation. In comparison with these studies, in the present laboratory study no correlation was observed between allometric data and filtration rate, although a bigger sample size would be recommended to achieve a more reliable outcome.

In the experimental study, temperature was set in accordance with optimal temperature range for freshwater naiads. However, other intrinsic factors like individual filtration rates during the exposure to the inoculum may had introduced some variability in the results.

Furthermore, in the present laboratory study we observed *Aeromonas* spp. growing in control samples from all exposure periods and other genera were not identified by *VITEK®2 system*. All samples, including negative control samples, corresponded to naiads coming from the captive breeding program from the *Consorci de l'Estany*, carried out under controlled conditions and in which no health problems or mortality events are identified. *Aeromonas* spp. was also found in sentinel naiads from the field sampling. In that sense, previous studies have investigated indigenous microbiota of bivalves. For example, *Aeromonas* and *Pseudomonas* seemed to be opportunistic pathogens being present in the tissue of zebra mussels subjected to thermal stress (Gu and Mitchell, 2002). Our data suggests that *Aeromonas* spp. found in analysed control naiads and sentinel naiads could belong to indigenous bacteria.

The present study indicates that freshwater naiads (*Unio mancus*) may be a good model for the bio-monitoring of AMR-Enterobacteriaceae in freshwater systems and thus, be used as sentinels of anthropogenic pressure. Efforts on calculating filtration rates for this species and minimally required density of naiad's population for this purpose could provide a better assessment of the cost-benefit analyses and its suitability to control AMR environmental contamination. Overall, further research on these topics will be required to give a boost to naiad's repopulation in river basins.

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## 7. Annexes


**Figure 1.** Geographical distribution of *Unio mancus* in dark green color (*IUCN*).


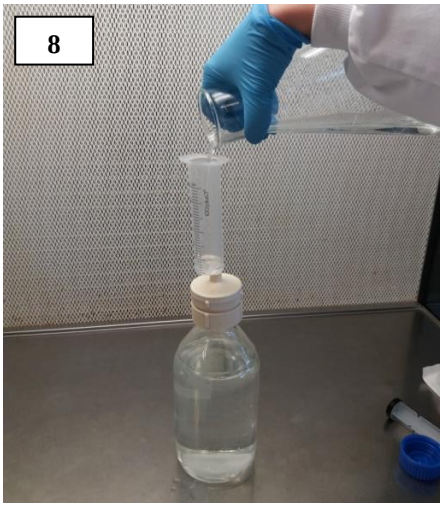


**Figure 3.** Juvenile naiads in a grid box from the breeding centre *Consorci de l'Estany*.

**Figure 8.** Water filtration process. The membrane filter is situated between the syringe and the glass bottle.


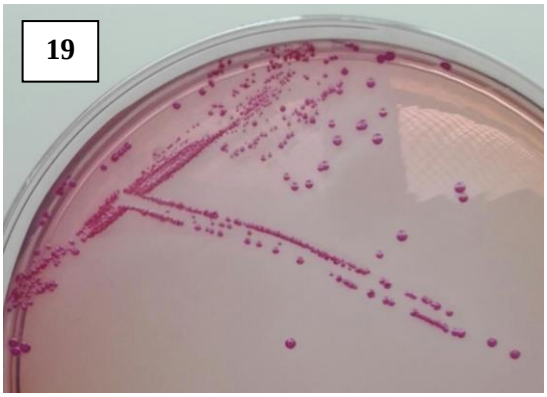


**Figure 18.** Details of *Klebsiella* spp. (circled in blue) and *E. coli* (circled in red) colonies on McConkey medium. *Klebsiella* spp. colonies grow in a dark pink colour, whereas *E. coli* colonies grow in dark red colour. Relatively small and pale colonies growing across the whole plate were identified as *Aeromonas* spp. in every sampling of the study. **Figure 19.** Details of an (resistant) *E. coli* pure culture in McConkey medium supplemented with ceftriaxone (2mg/L).

**Table 5-10**. Minimal inhibitory concentrations of bacterial isolates of sentinel naiads from the *L'Estany de Banyoles* lake and its scraping irrigations. Tested concentrations for each antimicrobial are painted in white. Blue colour boxes indicate ECOFF values non-determined by EUCAST. Note that samples classified as "larger than a specific concentration" are indicated in red in the same box than samples with the specific concentration.

[1]ECOFF non determined by EUCAST. [2]Total resistant.

| ***Citrobacter freundii* (n=6)** | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Antimicrobial** | **ECOFF (µg/mL)** | **Concentrations (µg/mL)** | | | | | | | | | | | | | | | | | **Total R[2]** |
| | | **<0.015** | **0.015** | **0.03** | **0.06** | **0.125** | **0.25** | **0.5** | **1** | **2** | **4** | **>8** | **16** | **32** | **>64** | **>128** | **256** | **512** | **1024** | |
| **Gentamicin** | ≤2 | | | | | | 6 | | | 1 | | | | | | | | | | 0 |
| **Ampicillin** | ≤8 | | | | | | | 2 | | | | | | | 4 | | | | | 4 |
| **Cefotaxime** | ≤0.5 | | | | | 4 | | 1 | | 1 | | | | | | | | | | 1 |
| **Ceftazidime** | ≤1 | | | | | | 4 | | 1 | | | 1 | | | | | | | | 1 |
| **Meropenem** | ≤0.25 | | 5 | 1 | | | | | | | | | | | | | | | | 0 |
| **Chloramphenicol** | ND[1] | | | | | | | | | | 6 | | | | | | | | | |
| **Azithromycin** | ND[1] | | | | | | | | | | | 3 | | | | | | | | |
| **Ciprofloxacin** | ND[1] | 2 | | | 1 | 1 | | | | | | 1; **1** | | | | | | | | |
| **Nalidixic Acid** | ND[1] | | | | | | | | | 4 | | | | | | 1; **1** | | | | |
| **Sulfamethoxazole** | ND[1] | | | | | | | | | | 4 | | 2 | | | | | | | |
| **Trimethoprim** | ND[1] | | | | | 4 | | 1 | | 1 | | | | | | | | | | |
| **Tetracycline** | ND[1] | | | | | | | | 6 | | | | | | | | | | | |
| **Tigecycline** | ≤1 | | | | | 6 | | | | | | | | | | | | | | 0 |
| **Colistin** | ND[1] | | | | | | | 6 | | | | | | | | | | | | |

### *Enterobacter cloacae* (n=1)

| Antimicrobial | ECOFF (µg/mL) | Concentrations (µg/mL) | | | | | | | | | | | | | | | | | Total R[2] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | <0.015 | 0.015 | 0.03 | 0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | >64 | 128 | 256 | 512 | 1024 | |
| **Gentamicin** | ≤2 | | | | | | 1 | | | | | | | | | | | | | 0 |
| **Ampicillin** | ND[1] | | | | | | | | | | | | 1 | | | | | | | |
| **Cefotaxime** | ≤0.5 | | | | | 1 | | | | | | | | | | | | | | 0 |
| **Ceftazidime** | ≤1 | | | | | | 1 | | | | | | | | | | | | | 0 |
| **Meropenem** | ≤0.125 | | 1 | | | | | | | | | | | | | | | | | 0 |
| **Chloramphenicol** | ND[1] | | | | | | | | | | 1 | | | | | | | | | |
| **Azithromycin** | ND[1] | | | | | | | | 1 | | | | | 1 | | | | | | |
| **Ciprofloxacin** | ≤0.125 | 1 | | | | | | | | | | | | | | | | | | 0 |
| **Nalidixic Acid** | ND[1] | | | | | | | | | 1 | | | | | | | | | | |
| **Sulfamethoxazole** | ND[1] | | | | | | | | | | 1 | | | | | | | | | |
| **Trimethoprim** | ND[1] | | | | | 1 | | | | | | | | | | | | | | |
| **Tetracycline** | ≤16 | | | | | | | | 1 | | | | | | | | | | | 0 |
| **Tigecycline** | ≤2 | | | | | 1 | | | | | | | | | | | | | | 0 |
| **Colistin** | ≤2 | | | | | | | 1 | | | | | | | | | | | | 0 |

### *Escherichia coli* (n=18)

| Antimicrobial | ECOFF (µg/mL) | Concentrations (µg/mL) | | | | | | | | | | | | | | | | | Total NWT[2] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | <0.015 | 0.015 | 0.03 | 0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | >4 | >8 | 16 | >32 | >64 | >128 | 256 | 512 | >1024 | |
| **Gentamicin** | ≤2 | | | | | | 17 | | | 1 | | | | **1** | | | | | | 1 |
| **Ampicillin** | ≤8 | | | | | | | 2 | | 6 | 2 | | | | **8** | | | | | 8 |
| **Cefotaxime** | ≤0.25 | | | | | 13 | | | | 1 | 2;**2** | | | | | | | | | 5 |
| **Ceftazidime** | ≤0.5 | | | | | | 15 | | | | | 2;**1** | | | | | | | | 3 |
| **Meropenem** | ≤0.125 | | 15 | | 1 | 1 | 1 | | | | | | | | | | | | | 1 |
| **Chloramphenicol** | ≤16 | | | | | | | | | | 16 | | | | 1 | **1** | | | | 2 |
| **Azithromycin** | ND[1] | | | | | | | | 11 | | 4 | 3 | | | | | | | | |
| **Ciprofloxacin** | ≤0.064 | 9 | | 2 | 1 | | 2 | 2 | | | | 2 | | | | | | | | 6 |
| **Nalidixic Acid** | ≤16 | | | | | | | | | 13 | | | | 1 | | 2;**2** | | | | 5 |
| **Sulfamethoxazole** | ≤64 | | | | | | | | | | 6 | | 2 | 7 | | | | | **3** | 3 |
| **Trimethoprim** | ≤2 | | | | | 11 | | | 2 | | 2 | | | **3** | | | | | | 5 |
| **Tetracycline** | ≤8 | | | | | | | | 15 | 1 | | | | | 1;**1** | | | | | 2 |
| **Tigecycline** | ≤0.5 | | | | | 18 | | | | | | | | | | | | | | 0 |
| **Colistin** | ≤2 | | | | | | | 18 | | | | | | | | | | | | 0 |

***Klebsiella oxytoca* (n=9)**

| Antimicrobial | ECOFF (µg/mL) | Concentrations (µg/mL) | | | | | | | | | | | | | | | | | | Total $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | <0.015 | 0.015 | 0.03 | 0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | >32 | 64 | 128 | 256 | 512 | 1024 | |
| **Gentamicin** | ≤2 | | | | | | 9 | | | | | | | | | | | | | 0 |
| **Ampicillin** | ≤8 | | | | | | | | | | | 3 | 2 | 3 | 1 | | | | | 6 |
| **Cefotaxime** | ≤0.5 | | | | | 9 | | | | | | | | | | | | | | 0 |
| **Ceftazidime** | ≤1 | | | | | | 9 | | | | | | | | | | | | | 0 |
| **Meropenem** | ≤0.25 | | 9 | | | | | | | | | | | | | | | | | 0 |
| **Chloramphenicol** | ND[1] | | | | | | | | | | 9 | | | | | | | | | |
| **Azithromycin** | ND[1] | | | | | | | | 2 | | | 2 | 5 | | | | | | | |
| **Ciprofloxacin** | ND[1] | 6 | | 2 | | | 1 | | | | | | | | | | | | | 1 |
| **Nalidixic Acid** | ND[1] | | | | | | | | | 8 | | | | | | 1 | | | | |
| **Sulfamethoxazole** | ND[1] | | | | | | | | | | | | 1 | 5 | 3 | | | | | |
| **Trimethoprim** | ND[1] | | | | | 6 | | 2 | | | | | | **1** | | | | | | |
| **Tetracycline** | ND[1] | | | | | | | | 9 | | | | | | | | | | | 0 |
| **Tigecycline** | ≤1 | | | | | 9 | | | | | | | | | | | | | | 0 |
| **Colistin** | ND[1] | | | | | | | 9 | | | | | | | | | | | | 0 |

***Klebsiella pneumoniae* (n=8)**

| Antimicrobial | ECOFF (µg/mL) | Concentrations (µg/mL) | | | | | | | | | | | | | | | | | | Total $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | <0.015 | 0.015 | 0.03 | 0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | >4 | >8 | 16 | 32 | 64 | 128 | 256 | 512 | >1024 | |
| **Gentamicin** | ≤2 | | | | | | 9 | | | | | | | | | | | | | 0 |
| **Ampicillin** | ≤8 | | | | | | | | | | | 1 | 2 | 2 | 1;**2** | | | | | 6 |
| **Cefotaxime** | ≤0.25 | | | | | 7 | | | | | **1** | | | | | | | | | 0 |
| **Ceftazidime** | ≤0.5 | | | | | | 6 | 1 | | | | **1** | | | | | | | | 0 |
| **Meropenem** | ≤0.125 | | 8 | | | | | | | | | | | | | | | | | 0 |
| **Chloramphenicol** | ND[1] | | | | | | | | | | 8 | | | | | | | | | |
| **Azithromycin** | ND[1] | | | | | | | | 1 | | | 4 | 3 | | | | | | | |
| **Ciprofloxacin** | ≤0.125 | 1 | 1 | 5 | | | | 1 | | | | | | | | | | | | 1 |
| **Nalidixic Acid** | ≤ND[1] | | | | | | | | | 8 | | | | | 1 | | | | | |
| **Sulfamethoxazole** | ≤ND[1] | | | | | | | | | | 1 | | 1 | 4 | 1 | | | | **1** | |
| **Trimethoprim** | ≤ND[1] | | | | | 1 | | 5 | 1 | 1 | | | | | | | | | | |
| **Tetracycline** | ≤8 | | | | | | | | 6 | 1 | | | | | **1** | | | | | 0 |
| **Tigecycline** | ≤2 | | | | | 8 | | | | | | | | | | | | | | 0 |
| **Colistin** | ≤2 | | | | | | | 8 | | | | | | | | | | | | 0 |

***Salmonella spp.* (n=1)**

| Antimicrobial | ECOFF (µg/mL) | Concentrations (µg/mL) | | | | | | | | | | | | | | | | | Total $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **<0.015** | **0.015** | **0.03** | **0.06** | **0.125** | **0.25** | **0.5** | **1** | **2** | **4** | **8** | **16** | **32** | **>64** | **128** | **256** | **512** | **1024** | 0 |
| **Gentamicin** | ≤2 | | | | | | 1 | | | | | | | | | | | | | 1 |
| **Ampicillin** | ≤8 | | | | | | | | | | | | | | 1 | | | | | 0 |
| **Cefotaxime** | ≤0.5 | | | | | 1 | | | | | | | | | | | | | | 0 |
| **Ceftazidime** | ≤2 | | | | | | 1 | | | | | | | | | | | | | 0 |
| **Meropenem** | ≤0.125 | | 1 | | | | | | | | | | | | | | | | | 0 |
| **Chloramphenicol** | 16 | | | | | | | | | | 1 | | | | | | | | | |
| **Azithromycin** | ND[1] | | | | | | | | | | 1 | | | | | | | | | 0 |
| **Ciprofloxacin** | ≤0.064 | 1 | | | | | | | | | | | | | | | | | | 0 |
| **Nalidixic Acid** | ≤16 | | | | | | | | | 1 | | | | | | | | | | |
| **Sulfamethoxazole** | ND[1] | | | | | | | | | | | | | | | | | 1 | | 0 |
| **Trimethoprim** | ≤2 | | | | | | | | | 1 | | | | | | | | | | 0 |
| **Tetracycline** | ≤8 | | | | | | | | 1 | | | | | | | | | | | |
| **Tigecycline** | ND[1] | | | | | | | 1 | | | | | | | | | | | | 0 |
| **Colistin** | ≤16 | | | | | | | 1 | | | | | | | | | | | | 0 |