Ecotoxicology and Environmental Safety 120 (2015) 235–242

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Detection and diversity of aeromonads from treated wastewater and fish inhabiting effluent and downstream waters ☆

Natalija Topic Popovic [a,*], Snjezana P. Kazazic [b], Ivancica Strunjak-Perovic [a], Josip Barisic [a], Roberta Sauerborn Klobucar [a], Slavko Kepec [c], Rozelinda Coz-Rakovac [a]

[a] Laboratory for Ichthyopathology-Biological Materials, Rudjer Boskovic Institute, Zagreb, Croatia
[b] Laboratory for Chemical Kinetics and Atmospheric Chemistry, Rudjer Boskovic Institute, Zagreb, Croatia
[c] Virkom d.o.o., Public Water Supply and Wastewater Services, Virovitica, Croatia

ARTICLE INFO

Article history:
Received 22 January 2015
Received in revised form
27 May 2015
Accepted 8 June 2015
Available online 16 June 2015

Keywords:
WWTP
Aeromonas
MALDI TOF MS
API 20E
Wastewater

ABSTRACT

A two-season investigation of the wastewater treatment plant (WWTP) effluent, of related waters, sludge and fish across a wide area and 11 stations, with emphasis on *Aeromonas* spp. was conducted. *Aeromonas veronii* was the prevailing aeromonad isolated by MALDI TOF MS in the summer period. A rise of *Aeromonas hydrophila* was observed in summer in raw sewage, treated wastewater and effluent-carrying canal. The ratio of aeromonad species retrieved from fish tissues did not correspond with the water and sludge findings, as in spring in the effluent-carrying canal fish carried *Aeromonas salmonicida* ssp. *salmonicida* and *Aeromonas bestiarum*, while in summer mainly *A. veronii* and *Acinetobacter johnsonii* were isolated from fish tissues in the same location. No correlation was established between fecal coliforms/enterococci and aeromonad occurrence. All retrieved *Aeromonas* species demonstrated a distinct spectral pattern, with peaks showing unique mass distribution ranging from 4000 to 10,000 Da. Hierarchical clustering separated aeromonads of all isolated species and clustered closely related strains together. Resistance was determined towards amoxicillin, and frequently towards sulfamethoxazole and erythromycin. In summer, a high proportion of water and sludge *Aeromonas* species demonstrated multiple resistance patterns towards five or more antimicrobials. The quinolone resistance of water aeromonads was mostly related to *A. veronii*. There are potential health concerns regarding aeromonad exposure amongst recreational fishermen who come into contact with fish inhabiting waters downstream from the WWTP, and WWTP workers who are occupationally exposed to wastewaters and their aerosols.

1. Introduction

Mesophilic aeromonads have been found in almost every aquatic environment, including chlorinated drinking water, raw sewage, groundwater and both polluted and unpolluted streams and rivers (Huddleston et al., 2006). Members of the genus *Aeromonas* are symbionts of zebrafish, leeches, and dreissenid mussels (Skwor et al., 2014). Aeromonads have been associated with bacterial zoonoses and fish diseases (Austin and Austin, 1999), and recovered from urban sewage and rivers with sewage pollution (Vandewalle et al., 2012). *Aeromonas caviae* was determined as a dominant species in waters contaminated by sewage and wastewater and pointed out as a potential indicator of sewage pollution (Ramteke et al., 1993). In the sewage treatment ponds aeromonad and coliform distribution is reported to have seasonal cycles, the amplitude of which increases further from the wastewater source (Monfort and Baleux, 1990).

Urban wastewater treatment plants (WWTP) were originally designed to reduce the biological oxygen demand, total suspended solids and nitrogen and phosphorus pollution, while the removal of pathogenic microorganisms has received less attention (Lucas et al., 2014). Although the primary and secondary treatments are able to remove up to 99% of fecal indicator bacteria (Servais et al., 2007; Lucas et al., 2014), the quality required to use treated wastewaters might be insufficient to achieve the level required for irrigation and recreational activities in the receiving water bodies.

The microbial quality of effluent water is a very important consideration, among other issues because it is reflected in the microbial flora of fish living downstream, which might be fished

☆This work was supported by the Croatian MSES Grant no. 098-1782739-249, and carried out in accordance with the EC Directive 86/609/EEC for animal experiments. Fish were manipulated by the competent authorized persons (licenced veterinarians) in accordance with the provisions of national legislation and the Institute's Bioethical Committee (No. BEP-274/2-2012). The authors declare no conflict of interest.
* Corresponding author.
E-mail address: ntopic@irb.hr (N. Topic Popovic).

http://dx.doi.org/10.1016/j.ecoenv.2015.06.011
0147-6513/

out recreationally and serve as a protein source for humans; however, aeromonads are mostly not considered in such estimations. The main concern in the nowadays customary use of treated wastewaters for fish farming and crop irrigation (El-Shafai et al., 2004; Piveli et al., 2008) is a public health risk. With that in mind, it is worthwhile to consider a broader aspect of the water quality along with fecal contaminants, and include other potentially pathogenic bacteria in this determination, particularly aeromonads. Also, antibiotic sensitivity of environmental *Aeromonas* spp. needs to be further addressed, since they have not been studied to the same extent as the clinical isolates (Goni-Urriza et al., 2000; Huddleston et al., 2006).

A two-season (spring and summer) microbiological investigation was conducted on representative water, sludge, and fish samples related to a WWTP processing municipal, hospital and sugar plant wastewaters, from 11 stations. The WWTP is a mechanical and chemical–biological facility with activated sludge, encompassing primary and secondary treatments of influents, treating primarily municipal wastewater deriving from a small city of 20,000 residents. Although in some cities wastewater from hospitals is pretreated or biologically treated on-site, on this location it is connected directly to a municipal sewer and treated at the municipal WWTP. The sugar plant is a significant contributor to the wastewater to be treated at the WWTP. The objective of this work was thus to conduct a two-season microbiological investigation of the WWTP effluent and related waters and sludge across a wide area of stations, with special emphasis on diversity *Aeromonas* species, their impact on health status of fish living downstream, and rapid discrimination with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) compared with the conventional identification methods.

2. Materials and methods

2.1. Study area

The study was carried out in spring and summer 2014. The samplings were conducted throughout the treatment process of a Croatian municipal WWTP, also receiving hospital and sugar plant wastewaters. Sugar plant was active in spring and inactive in summer. The WWTP treatment includes primary and secondary processes, including settling tanks, grit chambers, activated sludge biological process, aeration tanks, secondary tanks for removing the biomass and other suspended particles. The resultant final treated effluent is discharged into a natural water canal. This canal further downstream receives additional communal treated water from a biological treatment plant serving a small suburb, widens to enter a County canal which eventually ends up in Drava river. Therefore, sampling sites for water and sludge are defined as follows: 1: unaffected stream, not related to any industrial nor agricultural waters, considered as a reference site; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant; 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the Drava river; 10: WWTP active sludge; 11: sludge from the sugar plant depot (Fig. 1). Water and sludge were collected in glass sterile bottles and polypropylene flasks, refrigerated transported to the lab and immediately analyzed. Samplings were conducted at identical timepoints.

2.2. Sampling and animals

This work has been carried out in accordance with the EC Directive 86/609/EEC for animal experiments, and fish were manipulated by the competent authorized persons (licenced veterinarians) in accordance with the provisions of national legislation. Fish were caught by nets and angling at three locations depicted in Fig. 1: unaffected stream (site 1), canal receiving the effluent (site 5), County canal (site 9). In spring, 24 Prussian carp (*Carassius gibelio*) of both sexes were subjected to examination (mean weight 498.80 ± 232.04 g, mean length 213.46 ± 66.94 mm). In summer, 19 Prussian carp of both sexes (mean weight 112.94 ± 65.64 g, mean length 173.78 ± 30.35 mm) were examined. Specimens were randomly sampled, transported live to the laboratory and within few hours from the capture sacrificed by overdose of tricaine

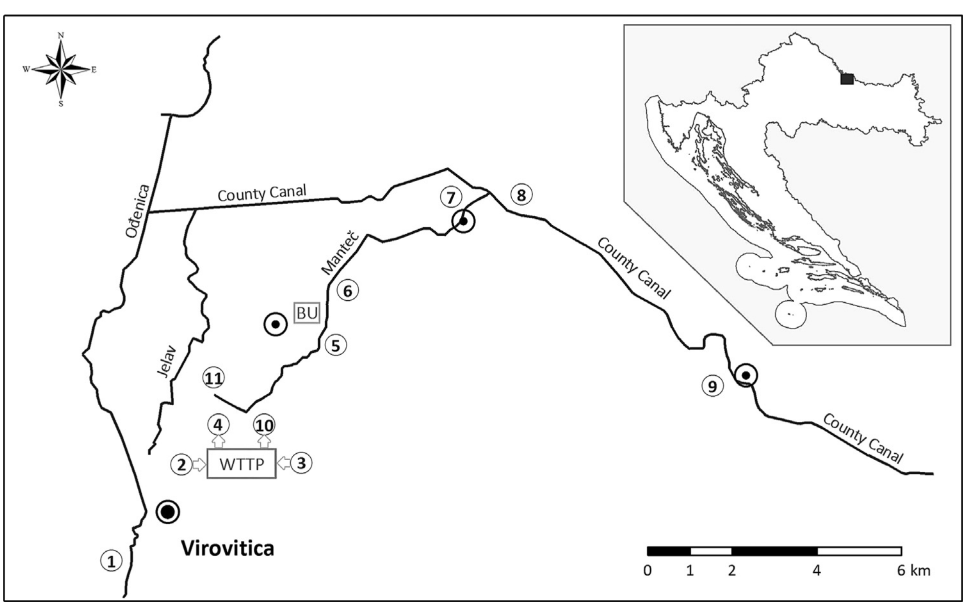

Fig. 1. Sampling sites for raw water, treated water and sludge drawn in the ArcGIS 10.1 program (location: NE Croatia) 1: unaffected stream, not related to any industrial nor agricultural waters, considered as a reference site; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant; 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the river; 10: WWTP active sludge; 11: sludge from the depot. Symbols: BU (biological treatment plant unit), circumpunct (inhabited area).