Aquatic bacterial contamination associated with sugarplant sewage outfalls as a microbial hazard for fish

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Abstract

The aim of the study was to compare bacterial composition and load in waters and fish related to the wastewater treatment plant (WWTP), particularly waters and wild fish affected by sugarplant processing (sugar cane and sugar beet). Aeromonads were the most frequently isolated group from water and fish. *A. hydrophila* was a prevailing species in isolates from water, followed by *A. veronii*, *Rheinheimera soli* and *Ochrobactrum anthropi*. Of indicator bacteria for aquatic

contamination from fish tissues, the most prominent were *V. cholerae*, *Enterobacter cloacae* and *E. sakazakii*. Sugar cane processing contributed to high viable cell counts at 37 °C while sugar beet processing contributed to high bacterial counts at 22 °C. Heterotrophs from gills of effluent fish were highest during sugar cane processing. Counts retrieved from fish skin were more uniform between effluent fish and fish from downstream waters. Antimicrobial resistance of bacteria isolated from water was high against amoxicillin, sulfamethoxazole, flumequine, norfloxacin and oxolinic acid in samples from the inflow of raw municipal wastewaters to WWTP, while resistance found in bacteria from the inflow of sugarplant mostly related to sulfamethoxazole and amoxicillin. The PCA analysis associated the occurrence of high heterotroph counts, *P. aeruginosa*, and intestinal enterococci on skin and gills with sugar cane, and yeasts and molds with sugar beet processing. Fish living in treated wastewaters and related water bodies could pose a microbial hazard if fished for human consumption, possibly causing infection when being handled and processed, as a risk of human pathogens penetrating fish tissues.

Keywords: Wastewater treatment plant · Fish · Bacteria · Resistance · Pollution

INTRODUCTION

Wastewater treatment plants (WWTPs) perform primary and secondary biological treatment of municipal and related waters, and sometimes tertiary treatment for agricultural irrigation and wetlands restoration. The complex microbial community found in the treated effluent of WWTPs, although significantly reduced (particularly in fecal indicator bacteria),

might still contain pathogenic bacteria which present a threat to fish living downstream (Topić Popović et al. 2015a). If used for human consumption, such fish may pose a potential public health risk.

When reared in treated domestic wastewater, which is not a rare practice due to the abundance of nutrients, silver carp (*Hypophthalmichthys molitrix*), common carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*) show sensitiveness to the impaired environment (Buras et al. 1987). Bacteria can be retrieved from their internal organs and tissues up to concentrations of 10^9 g^{-1}. If rearing stocking juveniles in treated wastewaters, and transferring them later to the regular fish farms, fish will likely reduce the numbers of bacteria, and the danger of pathogen transfer to humans would be avoided (Niewolak & Tucholski 2000). However, wild fish living in treated wastewaters and related water bodies which are fished for recreational purposes and human consumption, could pose a threat. The dominant fish species in slowly running lowland watersheds in Europe and Asia is Prussian carp (*Carassius gibelio*) (Lusk et al. 2010). It invaded European ponds, eutrophic lakes, canals, and small water reservoirs (USFWS 2012) due to its ability to grow and reproduce rapidly. It tolerates well the impaired environmental conditions, such as high organic loads or low levels of dissolved oxygen, and is a highly possible catch of recreational fishermen.

There are number of potential bacterial pathogens that might be related with contaminated waters from which representative bacterial indicators of human and (aquatic) animal contamination are chosen for screening. They include total and fecal coliforms, *Escherichia coli*, fecal streptococci and enterococci, *Salmonella* sp., *Shigella* sp., and *Vibrio* sp. (Naidoo & Olaniran 2014). However, indicator bacteria should always be assessed in the context of the study, taking into account the natural microbial ecology, biotic and abiotic physical-

chemical factors which could influence microbial growth. Negative indicator tests cannot guarantee the absence of a microbial hazard (Tortorello 2003). Also, sugarplants are significant contributors to the WWTP load, with high water demand and organic pollution (Ingaramo et al. 2009). For that reason, and in order to improve our understanding in assessing the biological risks for the fish living in the WWTP effluent and waters related to the WWTP, it is important to determine their bacterial community and diversity differences (Topić Popović et al. 2015a, b). With this objective, bacteria were identified at two seasonal time points from: (i) wastewaters from a Croatian municipal WWTP which also processes waters from a sugarplant, (ii) waters further downstream, from a wider canal which drains into the river Drava; (iii) wild Prussian carp inhabiting effluent-receiving waters and further downstream waters, in spring and fall. The hypothesis of the study was that both ubiquitous and pathogenic bacteria would be retrieved from fish tissues, in relation to season and activity of the sugarplant. The aim was to compare bacterial composition and load in different WWTP-related waters, and various fish tissues over seasons, as well as the occurrence of resistance to eight antimicrobial drugs tested.

MATERIALS AND METHODS

Study site

The study was conducted in spring and fall throughout the treatment process of a Croatian municipal WWTP, which also receives hospital and sugarplant wastewaters (Fig. 1). Sugarplant was active in spring (pre-washed sugar cane processing) and fall (sugar beet

processing) with 15.94 % and 30.83 % of treated waters, respectively. The final treated effluent outflows into a natural canal (location 1), which widens to enter a further downstream canal (location 2) draining into the river Drava. Fish and water were sampled from locations 1 and 2. Water was also sampled at the inflow of raw municipal wastewaters to the WWTP (location 3) and the inflow of sugarplant wastewaters to the WWTP (location 4).

Sample collection

The study was carried out in accordance with the EC Directive 86/609/EEC for animal experiments, and according to the Institute's directions for animal tests. Prussian carp (*Carassius gibelio*) were caught by nets and angling: in spring (n = 24) mean weight (W) 498.80 g ± 232.04 SD, mean length (L) 213.46 mm ± 66.94 SD; in fall (n = 45) W 127.80 g ± 97.32 SD, L 170.22 mm ± 45.32 SD. Fish were transported live to the laboratory and sacrificed by overdose of tricaine methane-sulfonate (MS-222, Sigma, St. Louis, Missouri, USA). Tissues (gills, anterior kidney) were fixed in 4 % neutral buffered formaldehyde, dehydrated through a graded ethanol-xylene series, embedded in paraplast, and stained with hematoxylin/eosin.

Analytical methods

Physico-chemical characteristics of water were analyzed according to the international standards as follows: determination of electrical conductivity, pH, suspended solids, dissolved oxygen, permanganate index, chemical oxygen demand (COD), biochemical oxygen demand after n days (BODn), dilution and seeding with allylthiourea, phosphorus with spectrometric

116 method, nitrite, total nitrogen by persulfate digestion method, cadmium reduction, and nitrate by
117 colorimetry (ISO 7888:1985, ISO 10523:2008, ISO 872:2005, ISO 5813:1983, ISO 8467:1993,
118 ISO 15705:2002, ISO 5815:1989, ISO 7150-1:1984, ISO 6878:2004, ISO 6777:1984, SM 4500-
119 NO3-E, respectively).

120 Methods for detection and enumeration of *Escherichia coli*, coliforms and enterococci
121 from water and sludge were used according to the Detection and enumeration of *E. coli* and
122 coliform bacteria – Part 1: Membrane filtration method (ISO 9308-1:2000/Corr.1:2008) and
123 Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method (ISO
124 7899-2:2000). *E. coli*, coliforms and enterococci were also measured from fish tissues (skin and
125 gill scrapings). All samples were inoculated on general purpose media and media for the
126 selective isolation of bacteria (all Oxoid Ltd, Basingstoke, England, UK). Samples of fish gills
127 and internal organs (kidney and liver) were streaked onto Tryptone Soya Agar, MacConkey Agar
128 (Oxoid) and Blood Agar (Certifikat doo, Osijek, Croatia). Colonies were subjected to
129 morphological, physiological and biochemical tests. The taxonomic position of the isolates was
130 determined by the MALDI Biotyper using MALDI-TOF (Matrix Assisted Laser Desorption
131 Ionization-Time of Flight) Mass Spectrometry (Bruker Daltonik GmbH, Bremen, Germany). The
132 ethanol/formic acid extraction was applied for MALDI TOF MS sample preparation as described
133 in Topić Popović et al. (2015a, b). Recorded mass spectra were processed with the MALDI
134 Biotyper 3.0 software package (Bruker Daltonik), using standard settings.

135 Antimicrobial susceptibility of the isolated strains was determined with Kirby-Bauer disk
136 diffusion method on Mueller Hinton agar (all Oxoid). The following antimicrobials with
137 respective concentrations were used in the test: oxytetracycline (OTC, 30 μg), amoxicillin

(AMC, 30 µg), oxolinic acid (OA, 2 µg), erythromycin (E, 15 µg), sulfamethoxazole (SMX, 50 µg), florfenicol (FFC, 30 µg), norfloxacin (NOR, 10 µg), flumequine (UB, 30 µg).

Statistical analysis

Statistical analyses were performed using SigmaPlot and SigmaStat Statistical Software ver. 11.0 (Jandel Corp. San Rafael, CA). Bacterial counts data were subjected to logarithmic transformation. All data were analyzed by multivariate analysis in order to extract variables or important related information, to identify possible clusters, and to identify trends between samples and/or variables.

In order to examine the possibility of viewing data sets independently of sampling site and processed sugar cane/sugar beet, t-test was used. Although the test showed that there was no significant difference between the values in the different canals ($p = 0.10$), bacterial composition in each sampling site and material processed was a significant factor: (i) effluent-receiving canal (CE) beet *vs*. cane, $p = 0.028$ and (ii) downstream county canal (CC) beet *vs*. cane, $p = 0.002$. So, for the further analysis, the data set was divided by sampling site (CE and CC) and processed material (B = beet and C = beet).

Also, the Pearson correlation test was conducted on the complete data matrix (physico-chemical parameters of water *vs*. bacteria, yeasts and molds on fish gills and skin, allowing the reduction of data. Analysis of possible data or dimension reduction is used in aquatic ecology as demonstrated by ter Braak and Verdonschot (1995).

Classification process was started with the Factor analysis (FA) which identified significant variables and assisted in reduction of the original data set. The Principal component

161 analysis (PCA) followed, using pattern recognition methods (Bosque-Sendra et al. 2012), in
162 order to effectively reduce redundant information (Sweidan et al. 2015). The Discriminant
163 analysis (DA) was used to evaluate the classification and to distinguish variables in relation to
164 season and location.
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167 **RESULTS**
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169 **Physico-chemical characteristics of water**
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171 Many water quality parameters measured at both fish sampling sites were higher or lower
172 than reference guidelines for cyprinid fish (Stoskopf 1993, Billard 1999). Dissolved oxygen and
173 oxygen saturation in spring and fall in canal receiving the final treated effluent were significantly
174 below listed for carp tolerance levels (Table S1. of the Supplement). During the activity of the
175 sugarplant, extreme values were noted at the inflow of sugarplant wastewaters to the WWTP for
176 suspended solids, chemical oxygen demand (COD), COD-Mn, and biochemical oxygen demand
177 (BODn). Ammonium, nitrite, nitrate and total nitrogen values during sugarplant activity were not
178 favorable for carp propagation.
179
180 **Microbial counts and species retrieved from water**
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182 Total viable cell counts and concentrations of bacteria, yeasts and molds from water at 4
183 sampling locations and three seasons are presented in Fig. S1 & S2 of the Supplement. Colony

counts at 22 °C were the highest at the inflow of sugarplant wastewaters to WWTP in fall (1.1 x 10^8 CFU mL^{-1}) and in spring (1.4 x 10^7 CFU mL^{-1}). Colony counts at 37 °C were the highest in spring at the same location (3.1 x 10^6 CFU mL^{-1}), which also yielded the highest loads of yeasts and molds (1.7 x 10^4 CFU mL^{-1} in fall, and 10^3 CFU mL^{-1} in spring, respectively). Fecal coliforms, intestinal enterococci, and *Pseudomonas aeruginosa* reached 6.76 x 10^6 CFU 100 mL^{-1}, 1.12 x 10^7 CFU 100 mL^{-1}, and 1.7 x 10^5 CFU 100 mL^{-1}, respectively, all in fall, at the inflow of raw municipal wastewater to WWTP. Sulphite-reducing clostridia and *E. coli* reached 3.5 x 10^3 CFU 100 mL^{-1}, and 5.5 x 10^5 CFU 100 mL^{-1} in fall at the inflow of sugarplant wastewaters, although the overall highest counts of *E. coli* were in fall at the inflow of raw municipal wastewaters to WWTP (8.5 x 10^5 CFU 100 mL^{-1}). *Listeria monocytogenes* was not isolated from any of the samples, but *L. inocua*, *L. grayi*, and *L. ivanovii* were retrieved in fall from the canal receiving the final treated effluent. Overall distribution of bacterial genera isolated from all water sampling locations is presented in Fig. S3. Aeromonads were the most frequently isolated group from most samples.

Microbial counts and species retrieved from fish tissues; tissue aberrations

Bacterial counts and concentrations of bacteria, yeasts and molds from skin and gills of fish are presented in Fig. S4 & S5. From gills, fecal coliforms and *E.coli* were not isolated, while from skin, fecal coliforms, *E.coli*, *P. aeruginosa*, and sulphite-reducing clostridia were not isolated. Colony counts from gills at 22 °C and 37 °C were highest in spring from effluent fish (location 1) (3.28 x 10^7 CFU mL^{-1}, and 2.22 x 10^7 CFU mL^{-1}, respectively). In fall, these counts did not reach over 7 x 10^5 CFU mL^{-1}, and 5.9 x 10^5 CFU mL^{-1}, respectively). The highest

measured yeasts, molds and *P. aeruginosa* concentrations on gills were in effluent fish in fall (832 CFU 100 mL^{-1}, 22 CFU 100 mL^{-1}, and 32 CFU 100 mL^{-1}, respectively). The same location also yielded the highest loads of intestinal enterococci on gills (in spring), 78 CFU 100 mL^{-1}. In all fish, the highest counts were in spring, at both temperatures, and the highest overall was from downstream canal (location 2) fish skin as 5.3 x 10^6 CFU 100 mL^{-1}. The highest yeasts and molds load from fish skin was in fall, from effluent fish (140 CFU mL^{-1}, and 17 CFU mL^{-1}, respectively). The concentration of yeasts and molds were several folds lower in skin than in gills, as well as bacterial counts at both temperatures. The highest intestinal enterococci concentration was in spring from fish skin of both canals. Overall distribution of bacterial genera isolated from fish gills and internal organs are presented in Fig. S6. From all tissues, aeromonads were the most prominent bacteria. The greatest diversity of bacterial species was found in fish from the downstream canal (location 2), particularly in gills. *A. hydrophila* was a prevailing species, followed by *A. veronii*, *Rheinheimera soli*, and *Ochrobactrum anthropi*. *E. coli* was not isolated from any of the internal fish tissues. Of indicator bacteria for aquatic contamination retrieved from internal tissues, the most prominent were *V. cholerae* (location 2 fish), *Enterobacter cloacae* and *Enterobacter sakazakii* (effluent fish).

Gill histopathology alterations included an increased number of bacteria and lymphocyte cells in a mucous matrix encompassing area between primary lamellae in effluent fish. The secondary lamellae in vicinity appear atrophic and necrotic (Fig. S7). Changes observed were severe, excluding possibility of functional respiration. In kidney sections of effluent fish epithelial necrosis of tubular lamina, exhibiting intratubular clumps were observed. These large aggregates were composed of necrotic debris within tubules, inflammatory and bacterial cells (Fig. S8).

Antimicrobial resistance of isolated strains

Antimicrobial resistance patterns of all bacterial strains isolated from water and fish tissues are presented in Fig. S9 & S10. Of bacteria isolated from water, the most prevalent resistance was observed in samples from the inflow of raw municipal wastewaters to WWTP (location 3), in spring against AMC and SMX; in summer against SMX, UB, NOR, and OA; in fall against AMC. Resistance against beta-lactam amoxicillin and sulfamethoxazole was established with high prevalence in most of the water samples irrespective of the type of water, and also for bacteria isolated from kidney and liver of effluent fish in spring (AMC) and from gills of effluent fish in fall (AMC, SMX). Downstream (location 2) fish bacteria were mostly intermediate or susceptible for tested antimicrobials.

Since aeromonads were the most represented of all bacterial genera, their resistance patterns are of special concern. Almost 63 % of fish aeromonads showed resistance against tested antimicrobials, and 50 % of them were resistant against SMX. All *Aeromonas* species from fish were resistant towards AMC. Some were also resistant against other drugs (E or SMX). *Aeromonas* species from water were also resistant against AMC, and 29 % of them showed multiple resistances against OA, E, OTC, and SMX.

PCA analyses

The relation and grouping of samples based on the monitored microbiological parameters in fish gills and skin was investigated. PCA analyses were conducted separately for the data on

253 gills and skin. For each data set a separate data matrix was built. For gills the matrix included 7
254 parameters (Counts at 37 °C, Counts at 22 °C, yeasts, molds, Enterococci, *P. aeruginosa*,
255 sulphite-reducing clostridia) and 6 for the skin (same as for the gills except *P. aeruginosa*). The
256 factor analysis was applied first in order to investigate possible reduction of observed parameters
257 and identify significant microbiological parameters. The PCA was conducted to observe the
258 score demonstrating groupings according to season, with their additional loading of significant
259 parameters (Fig. 2). The score plots show that the grouping was based on high percentage of the
260 explained variance in all cases (51.66 % for gills and 75.13 % for skin). The first principal
261 component (D1=29.87 %) for presented PCA of gills was under the influence of parameters
262 Counts at 22 and 37 °C, while the leading parameters in the second principal component
263 (D2=21.79 %) were molds, inversely proportional with yeasts parameter. The PCA plot (Fig.
264 2A) showed two possible outliers for samples in the canal receiving the effluent (CE) measured
265 in fall when sugar beet (B samples) was processed, and after processing of sugar cane (C
266 samples). The Grubbs test for outliers was applied on samples and outliers were confirmed. In
267 Figure 2B, the first and second principal components for the parameters isolated from fish skin
268 had the same leading parameters with addition of Enterococci in D1 (50.10 %) and in D2 (25.03
269 %), the inverted relationship belonging to molds, proportional to yeasts. PCA findings showed a
270 clear division of the samples depending on the processed material (beet or cane). All B samples
271 were grouped in the second and third quadrant.
272 The most important conclusion which results from Figure 2 is the percentage of the
273 explained variance for fish gills (51.66 %) and skin (75.13 %) which points to the fact that skin
274 microbiological analysis can significantly correlate when observed with the multivariate system.
275 Good grouping performance was an encouragement for testing if observed parameters allowed

discriminating the samples regarding the water sampling sites, so the discriminant analysis (DA) was applied. When the DA was conducted on all samples for the skin parameters, the success of classification based on the nine chosen parameters (dissolved oxygen, oxygen saturation, suspended solids, COD, COD-Mn, BODn, ammonium, nitrite and total phosphorus) was 85 %. However, when values for S-R clostridia were left out, the classification mounted to 99.25 % (Fig S11), particularly for waters after processed sugar cane. Fig. S11 thus confirms the high classification in the DA analysis. Knowing that fish skin data gave the most informative characteristic in this study, those values were related to physico-chemical characteristics of water. To reduce the number of observed variables, Pearson correlation test was conducted to identify the most important parameters (with a significance level α=0.05). For both processed waters (cane and beet) seven significant characteristics of water were identified, and correlation map revealed the significance of yeasts, enterococci and counts at 37 °C on fish skin. PCA analysis conducted on these inputs resulted with a Biplot which explains 85.77 % of all variations in the data set (Fig. 3).

DISCUSSION

The sugar processing industry is among those with the largest water demands and remains an important factor for the organic pollution (Ingaramo et al. 2009). Waters affected by the sugarplant processing in this assay, particularly the canal receiving the treated effluent as a fish-bearing canal, displayed marked aberrations from guidelines for cyprinid fish (Billard 1999). Problems with toxic nitrogen-containing compounds such as ammonia, nitrite and nitrate were

299 particularly expressed during sugar beet processing. The most important physical factor of
300 wastewater in sugar processing is the total suspended solid content (Sahu & Chaudhari 2015),
301 which was extremely high during sugar cane processing. It also contributed to the high viable
302 cell counts at 37 °C, while sugar beet processing contributed to high bacterial counts at 22 °C
303 and high concentrations of sulphite-reducing clostridia, *E. coli*, yeasts, molds, coliforms and
304 enterococci in water. High numbers of mesophiles and yeasts at levels > 6 log CFU g^{-1} were
305 previously found for beet wastewaters (Robles-Gancedo et al. 2009), as well as enteric bacteria
306 (Mitchell & Funke 1982). Such a footprint of wastewaters has an impact on fish living in treated
307 waters and also in further downstream waters, particularly on their ability to cope with stress and
308 susceptibility to diseases.

309 Yeasts and molds, often used for estimation of organic pollution in wastewaters
310 (Shimomura-Shimizu & Karube 2010), were found in high concentrations on effluent fish gills in
311 fall, during beet processing. Although it was not proven how the types of wastewater and
312 treatment processes influence yeast proliferation (Yang et al. 2011), it seems that higher
313 concentrations were retrieved from sugar-rich waters. During beet sugar extraction, yeasts,
314 mesophiles and thermophiles are the most numerous microbiota, while beet-washing water is an
315 important source of contamination (Robles-Gancedo et al. 2009). There is a likelihood of the
316 presence of potentially pathogenic and toxicogenic fungi in fish from such waters, and when
317 fished out for human consumption, they might pose a health threat and remain in cooked tissues
318 in spite of the thermal treatment (Bien and Nowak 2014). Most waterborne fungi remain in spore
319 form and are a particular risk to immunocompromised patients (Olaolu et al. 2014).

320 Passing immunological barriers, bacteria may penetrate and colonize various tissues in
321 polluted aquatic environments (Niewolak & Tucholski 2000). Interestingly, although retrieved in

high numbers in water, in neither of seasons were fecal coliforms and *E.coli* isolated from gills and skin, nor *P. aeruginosa*, and sulphite-reducing clostridia from skin. That could be partially explained by shedding of mucus from fish skin as a natural defense mechanism to prevent colonization by bacteria (Suhalin et al. 2008). It was also demonstrated that *E. coli* is rarely recovered from carp tissues if its water concentration stays below10^4 CFU mL^{-1} (Buras et al. 1985). In this work, sugar beet wastewaters inflowing to WWTP and treated effluent waters had *E. coli* loads 10^5 and 10^4 CFU 100 mL^{-1}, respectively, which might explain its absence from fish tissues. Although fecal coliforms were not isolated from gills and skin, their high water levels could lead to contamination of internal organs and muscle tissue, posing a risk to consumers if exceeding 10^3 CFU 100 mL^{-1} (Harnisz & Tucholski 2010). Relatively low counts of intestinal enterococci and *P. aeruginosa* were recovered from gills in this work. Yet, Guzman et al. (2004) established that fish may carry fecal indicator bacteria to non-polluted waters for long retention periods, causing infection when handling or consuming fish. The total heterotrophic plate counts exceeding 10^4 CFU mL^{-1} of water could bring risk of human pathogens penetrating fish tissues (Harnisz &Tucholski 2010). Although in this work they were reaching up to 10^5 CFU mL^{-1} in the canal receiving the treated effluent in spring (sugar cane processing), counts on fish gills and skin were on average much higher than in their bearing waters. Indeed, the spring counts on fish skin even multiplied in downstream canal fish when compared to effluent fish. Possible explanation might be that fish tissues provide a good substrate for the growth of most heterotrophic bacteria, with compositional attributes that affect bacterial growth. Heterotrophs thus multiply in the sub-environments provided by skin surfaces and gill areas (ICMSF 1998). If fish from such waters were to be used for human consumption, care should be taken regarding

344 the limits for heterotrophs in fish eaten raw/cooked (10^4/10^6 CFU g^{-1}, respectively) (El-Shafai et
345 al. 2004).

346 Both in the effluent and downstream fish, bacteria retrieved in both seasons were *A.*
347 *hydrophila* and *A. veronii* (internal organs). *A. hydrophila* has a worldwide distribution, and is
348 recognized as a primary pathogen of fish, causing a stress-mediated disease condition where
349 mortalities are influenced by elevated water temperatures (Austin & Austin 2007).
350 Environmental strains of *A. hydrophila* produce less enterotoxins when cultured at 37 °C than at
351 28 °C, while clinical isolates behave *vice versa* (Igbinosa et al. 2012). Thus, strains producing
352 virulence factors at 37 °C have better odds as human pathogens. *A. veronii* is also a species
353 potentially very pathogenic to humans, having a broad aquatic host range. Along with *A.*
354 *hydrophila*, it has been recognized as the causal agent of fish mortalities in freshwater
355 ecosystems, causing epizootic ulcerative syndrome (Skwor et al. 2014). *A. veronii* was the most
356 frequently isolated bacteria from internal tissues of effluent fish in this study, in both seasons,
357 which coincides with our previous work (Topić Popović et al. 2015a). The major public health
358 concern thus is the wound infection with aeromonads among individuals who capture and handle
359 the fish (El-Shafai et al. 2004). Internal organs of effluent fish yielded counts of *Enterobacter*
360 *cloacae* and *E. sakazakii*, while from downstream canal fish *V. cholera* was isolated,
361 demonstrating that indicator bacteria for aquatic contamination were retrievable also from a
362 further downstream fish, and mostly during sugar beet processing (fall). Tissue aberrations in fall
363 included severe gill and kidney lesions with bacterial and inflammatory cell aggregates. Similar
364 findings observed Declercq et al. (2015) when challenging trout and carp with highly virulent
365 *Flavobacterium columnare* isolates, which led to a high number of eosinophillic granular cells.

High bacterial loads in water may have led to increased bacterial cells in kidney, including hematopoietic tissues and renal tubules, as in the work of Islam et al. (2008).

Bacteria resistant to antibiotics and antibiotic resistant genes in the aquatic environment are an emerging contaminant issue (Sharma et al, 2016). Most bacteria form water and fish revealed resistance against beta-lactams (amoxicillin, AMC) and sulfamethoxazole, SMX, irrespective of the season/sugarplant activity, although multiple resistance was also noted. The AMC resistance could partially be explained by relatedness of AMC with ampicillin, towards which aeromonads show intrinsic resistance (Harnisz & Tucholski 2010). The SMX resistance, found in a high percentage of isolated aeromonads, could be due to its poor performance if not in combination with trimethoprim (Goni-Urriza et al. 2000). The overall resistance pattern is most likely a consequence of previous exposure to antimicrobials and chemotherapeutics due to municipal and hospital discharge waters processed by the WWTP, and cannot be directly correlated with the sugarplant activity. The antimicrobial resistance in wastewater-related waters is an important factor for emerging infectious diseases, as antibiotic resistance genes may be easily disseminated and imposing selective pressures (Figueira et al. 2012; Pruden et al. 2012; Sharma et al, 2016).

Previous studies demonstrating the use of PCA analysis investigated water quality based on fish biomarkers and water quality degree classification (Sweidan et al. 2015), the application of exploratory and unsupervised/supervised chemometric methods on chromatographic data, using the composition for the characterization and authentication (Bosque-Sendra et al. 2012), in monitoring of complex mixtures of toxicants found in aquatic ecosystems on fish species and their oxidative stress biomarkers (Dzul-Caamal et al. 2016), and in identifying the link between trophic ecology and metal accumulation in marine fish (Le Croizier et al. 2016). As the

application of multivariate tools proved very effective, we used PCA for the first time to investigate the relation and grouping of samples based on the monitored microbiological parameters in gills and skin. It was found that high heterotroph counts, *P. aeruginosa*, and intestinal enterococci on both skin and gills can be associated with sugar cane processing, while yeasts and moulds were proven to correlate predominantly with sugar beet parameters.

In conclusion, fish living under impaired conditions caused by sugar beet and sugar cane processing can become contaminated with bacterial pathogens, yeasts and molds. Although the relation between water quality and contamination of fish tissues is frequently controversial (WHO 2006), penetration of bacteria to fish tissues is a threat. Thus safety measures during handling and processing of fish, often fished out by recreational fishermen from downstream waters, are highly needed to avoid cross-contamination.

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