GDAŃSK UNIVERSITY OF TECHNOLOGY

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DOCTORAL DISSERTATION

Title of PhD dissertation: Analysis of the impact of wastewater discharge on recipients: synergistic approach

Title of PhD dissertation (in Polish): Analiza wpływu zrzutu ścieków na odbiorniki – podejście synergiczne

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dr hab. inż. Aneta Łuczkiewicz, prof. PG	dr hab. Katarzyna Jankowska, prof. PG

Gdańsk, 2023

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DESCRIPTION OF DOCTORAL DISSERTATION

The Author of the doctoral dissertation: M.Sc. Agnieszka Kalinowska

Title of doctoral dissertation: Analysis of the impact of wastewater discharge on recipients: synergistic approach

Title of doctoral dissertation in Polish: Analiza wpływu zrzutu ścieków na odbiorniki – podejście synergiczne

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Summary of PhD dissertation in English:
The dissertation presents the analysis of chemical and microbial composition of wastewater and examines the impact of wastewater discharge on the environment. Various case studies were analysed: from small settlements in a pristine European Arctic, to large municipal wastewater treatment plants in areas subjected to greater anthropogenic pressure. In order to comprehensively analyse the impact of wastewater discharge on the receiving waterbody, a holistic approach, combining multiple analytical methods, was chosen. The results of the study show that despite high bacterial removal rates, wastewater treatment plants based on biological methods, still release significant amounts of microorganisms into the environment. Wastewater included bacteria typical to (1) activated sludge (associated with nitrogen cycling or activated sludge bulking) or (2) to the human digestive system (including those showing antibiotic resistance). The biochemical potential and taxonomic structure of the treated wastewater microbiome varied throughout the year, reflecting seasonal fluctuations of wastewater treatment efficiency. At the same time, higher values of prokaryotic cells number, prokaryotic biomass or average cell volume were found in environmental samples influenced by wastewater discharge, compared to reference points. Additionally, this thesis proposed and validated a method for monitoring the occurrence of beta-lactam antibiotic resistance among coliform bacteria. This method can be widely used to assess the spread of antibiotic resistance.

Summary of PhD dissertation in Polish:
W pracy dokonano analizy składu chemicznego i mikrobiologicznego ścieków oraz zbadano wpływ zrzutu ścieków na środowisko. Przeanalizowano różne studia przypadku: od małych osad w dziewiczym rejonie Arktyki Europejskiej, po duże oczyszczalnie ścieków komunalnych na obszarach poddanych większej presji antropogenicznej. W celu kompleksowej analizy wpływu zrzutu ścieków na odbiornik, wybrano podejście holistyczne, łączące wiele metod analitycznych. Wyniki pracy pokazują, że pomimo wysokiego stopnia usuwania bakterii, oczyszczalnie ścieków oparte na metodach biologicznych

uwalniają do środowiska znaczne ilości mikroorganizmów, w tym bakterie typowe dla osadu czynnego (związane z obiegiem azotu lub pęcznieniem osadu czynnego) lub występujące w układzie pokarmowym człowieka (w tym wykazujące cechy lekooporności). Potencjał biochemiczny oraz struktura taksonomiczna mikrobiomu ścieków oczyszczonych zmieniała się w ciągu roku, odzwierciedlając sezonowe wahania efektywności pracy oczyszczalni ścieków. Jednocześnie w próbkach środowiskowych będących pod wpływem zrzutu ścieków stwierdzano wyższe wartości całkowitej liczby komórek prokariotycznych, ich biomasy i średniej objętości, w porównaniu z punktami referencyjnymi. Dodatkowo w pracy zaproponowano i zweryfikowano metodę monitoringu występowania oporności na antybiotyki beta-laktamowe wśród bakterii z grupy coli. Metoda ta może być powszechnie używana do oceny zagrożeń związanych ze zjawiskiem lekooporności.

GDAŃSK UNIVERSITY OF TECHNOLOGY
Faculty of Civil and Environmental Engineering

M.Sc. Agnieszka Kalinowska

ANALYSIS OF THE IMPACT OF WASTEWATER DISCHARGE ON RECIPIENTS: SYNERGISTIC APPROACH

Analiza wpływu zrzutu ścieków na odbiorniki – podejście synergiczne

DOCTORAL DISSERTATION

Supervisor:

Ph.D., D. Sc., Eng. Aneta Łuczkiewicz

Auxiliary supervisor:

Ph.D., D. Sc. Katarzyna M. Jankowska

Gdańsk 2023

Pracę dedykuję wszystkim, którzy we mnie wierzyli
i wspierali mnie na jakimkolwiek etapie, w jakikolwiek sposób.

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List of Manuscripts

The following Manuscripts form the backbone of this thesis and are referred to the text by Roman numerals I-IV:

Manuscript I: **Kalinowska, A.,** Szopińska, M., Chmiel, S., Kończak, M., Polkowska, Ż., Artichowicz, W., Jankowska K., Nowak A. & Łuczkiewicz, A. (2020). Heavy Metals in a High Arctic Fiord and Their Introduction with the Wastewater: A Case Study of Adventfiorden-Longyearbyen System, Svalbard. Water, 12(3), 794. **IF=3.10**, https://doi.org/10.3390/w12030794

Manuscript II: **Kalinowska A**., Jankowska K., Fudala-Książek S., Pierpaoli M., Łuczkiewicz A., (2021), The microbial community, its biochemical potential, and the antimicrobial resistance of Enterococcus spp. in Arctic lakes under natural and anthropogenic impact, Science of the Total Environment, **IF=7.96**, https://doi.org/10.1016/j.scitotenv.2020.142998

Manuscript III: **Kalinowska, A.**, Pierpaoli, M., Jankowska, K., Fudala-Ksiazek, S., Remiszewska-Skwarek, A., & Łuczkiewicz, A. (2022), Insights into the microbial community of treated wastewater, its year-round variability and impact on the receiver, using cultivation, microscopy and amplicon-based methods, Science of the Total Environment, **IF=7.96**, https://doi.org/10.1016/j.scitotenv.2022.154630

Manuscript IV: Marano, R.B.M., Fernandes, T., Manaia, C.M., Nunes, O., Morrison, D., Berendonk, T.U., Kreuzinger, N., Telson, T., Corno, G., Fatta-Kassinos, D., Merlin, C., Topp, E., Jurkevitch, E., Henn, L., Scott, A., Heß, S., Slipko, K., Laht, M., Kisand, V., Di Cesare, A., Karaolia, P., Michael, S.G., Petre, A.L., Rosal, R., Pruden, A., Riquelme, V., Agüera, A., Esteban, B., Luczkiewicz, A., **Kalinowska, A.**, Leonard, A., Gaze, W.H., Adegoke, A.A., Stenstrom, T.A., Pollice, A., Salerno, C., Schwermer, C.U., Krzeminski, P., Guilloteau, H., Donner, E., Drigo, B., Libralato, G., Guida, M., Bürgmann, H., Beck, K., Garelick, H., Tacão, M., Henriques, I., Martínez-Alcalá, I., Guillén-Navarro, J.M., Popowska, M., Piotrowska, M., Quintela-Baluja, M., Bunce, J.T., Polo-López, M.I., Nahim–Granados, S., Pons, M.N., Milakovic, M., Udikovic-Kolic, N., Ory, J., Ousmane, T., Caballero, P., Oliver, A., Rodriguez-Mozaz, S., Balcazar, J.L., Jäger, T., Schwartz, T., Yang, Y., Zou, S., Lee, Y., Yoon, Y., Herzog, B., Mayrhofer, H., Prakash, O., Nimonkar, Y., Heath, E., Baraniak, A., Abreu-Silva, J., Choudhury, M., Munoz, L.P., Krizanovic, S., Brunetti, G., Maile-Moskowitz, A., Brown, C., Cytryn, E. (2020). A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. Environment International, 144, 106035. **IF=9.62,** https://doi.org/10.1016/j.envint.2020.106035

Contribution to Manuscripts

- **Manuscript I**: conceptualization, investigation, data curation, writing—original draft preparation, writing—review and editing, project administration, funding acquisition,
- **Manuscript II**: investigation, methodology, software, data curation, visualization, writing - original draft, writing - review and editing,
- **Manuscript III**: conceptualization, investigation, methodology, software, formal analysis, data curation, visualization, writing - original draft preparation, writing - review and editing
- **Manuscript IV**: validation, sample collection, microbial cultivation, data collection, verification and analysis on the side of Polish project partner - Gdańsk University of Technology

The points value and basic journal metric based on Impact Factor for each journal are listed in Table 1 below.

Table 1 Journal points based on the Polish Ministry of Science and Higher Education and Impact Factors.

Manuscript	Journal	Number of authors	Point value	Impact Factor (5 years)	Impact Factor (2022)
I	Water	9	70	3.229	3.103
II	Science of the Total Environment	5	200	7.842	7.963
III	Science of the Total Environment	6	200	7.842	7.963
IV	Environment International	85*	140	9.620	9.621
Total			610	28.533	28.650

*global research

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Streszczenie

Od ponad wieku oczyszczalnie ścieków odgrywają istotną rolę w ochronie zdrowia ludzkiego i w działaniu na rzecz poprawy jakości wód powierzchniowych, zarówno w aspekcie ograniczania ich eutrofizacji, jak i rozprzestrzeniania się chorób przenoszone drogą wodną. Jednak skład ścieków komunalnych jest bardziej złożony - zawierają one bowiem, m.in. środki higieny osobistej, środki czyszczące, piorące i hormonalne, a także farmaceutyki i ich metabolity, co stwarza nowe zagrożenia dla środowiska i wyzwania dla sektora gospodarki wodno-ściekowej. Na przykład, oczyszczalnie ścieków zaczęły być postrzegane jako rezerwuar determinant oporności, jednak skład społeczności mikrobiologicznej ścieków oczyszczonych jest nadal pomijany w badaniach. Nieznana jest również przeżywalność mikroorganizmów, ich adaptacja czy rola w środowisku.

W tym kontekście, niniejsza praca miała na celu wypełnienie luk w wiedzy dotyczących wpływu zrzutu ścieków na wody odbiornika. Ze względu na uniwersalność tego zagadnienia, badania obejmowały różne studia przypadków, znajdujące się w różnych lokalizacjach geograficznych: od ścieków generowanych przez małe osady położone w stosunkowo dziewiczym środowisku arktycznym, do dużych oczyszczalni ścieków komunalnych zlokalizowanych na obszarach miejskich, poddanych znacznie większej presji antropogenicznej. W celu kompleksowej analizy wpływu zrzutu ścieków na odbiornik, wybrano podejście holistyczne, łączące wiele metod analitycznych. Zastosowanie szerokiego spektrum technik laboratoryjnych miało na celu stworzenie synergicznej korzyści poprzez wzajemne wspieranie się metod, przy jednoczesnym niwelowaniu ich wad. Przyjęte podejście posłużyło również do sprawdzenia zaproponowanej metody monitorowania nowych wzorców oporności wśród bakterii z grupy coli.

Wyniki pracy pokazują, że pomimo wysokiego stopnia usuwania bakterii (≥99%, w zależności od zastosowanej technologii), oczyszczalnie ścieków oparte na metodach biologicznych nadal uwalniają do środowiska znaczne ilości mikroorganizmów. Ścieki oczyszczone zawierały bakterie typowe dla osadu czynnego (zarówno te biorące udział w obiegu azotu, jak i związane z pęcznieniem osadu czynnego) oraz bakterie związane z układem pokarmowym człowieka (w tym wykazujące cechy lekooporności). Potencjał biochemiczny oraz struktura taksonomiczna mikrobiomu ścieków oczyszczonych zmieniała się w ciągu roku, odzwierciedlając sezonowe wahania efektywności pracy oczyszczalni ścieków. Jednocześnie w próbkach środowiskowych będących pod bezpośrednim wpływem zrzutu ścieków oczyszczonych stwierdzano wyższe wartości całkowitej liczby komórek prokariotycznych, ich biomasy i średniej objętości, w porównaniu z punktami referencyjnymi. Wyższy był również stosunek komórek żywych do martwych.

Występowanie bakterii wykazujących cechy oporności odnotowano zarówno w ściekach oczyszczonych, jak i w wodach odbiornika. Stwierdzano również występowanie genów oporności oraz integronów, czyli elementy genetyczne zapewniające bakteriom m.in. adaptację do zmieniających się warunków środowiskowych. Uzyskane wyniki potwierdziły potrzebę rutynowego monitorowania ścieków w celu właściwej oceny zagrożeń związanych ze zjawiskiem lekooporności. Służy temu zaproponowana w pracy metoda badania oporności na antybiotyki beta-laktamowe wśród bakterii z grupy coli. Może być ona stosowana na całym świecie, przy minimalnym zapotrzebowaniu na specjalistyczną wiedzę, odczynniki czy zaawansowany sprzęt, stanowi bowiem niewielką modyfikację istniejącego systemu badań rutynowych.

Abstract

For more than a century, wastewater treatment plants (WWTPs) have played a vital role in protecting human health and improving surface water quality, limiting the outbreaks of waterborne diseases and eutrophication, caused by excessive emissions of nutrients. Nonetheless, the composition of municipal wastewater is much more complex nowadays, as it includes personal care products, various cleaning, laundry and hormonal agents, as well as pharmaceuticals and their metabolites. All that creates novel hazards and challenges for the environment, and both: health and wastewater treatment sectors. As an example, WWTPs has been recognized as antibiotic resistance dissemination hotspots. However, the microbial community composition of the treated wastewater is still overlooked and its variability, survival, adaptation or function in the environment after the release from WWTP remain largely unknown.

In this context, this thesis aimed to tackle several knowledge gaps: characterize the chemical and microbial composition of the wastewater and thus investigate the impact of the wastewater discharged to the surface waters. Due to the versatility of the topic, a variety of case studies in different geographical locations were analyzed: from wastewater generated by small settlements located in the relatively pristine Arctic environment, to large municipal wastewater treatment plants located in urban areas subjected to a much larger anthropogenic pressure. In order to comprehensively analyze the impact of wastewater discharge on the receiving environment, a holistic approach combining a variety of analytical methods was chosen. The use of a wide range of laboratory techniques was intended to create a synergistic benefit by the mutual support of the methods, while offsetting their disadvantages. This approach was also used to validate the proposed method for monitoring the emerging resistance patterns among coliform bacteria.

The results showed that despite high bacterial removal rate (≥99%, depending on the type of treatment), WWTPs based on biological treatment still release significant amounts of microorganisms into the environment. WWTP effluent contains nutrient-cycling, activated sludge-bulking or human gut-related bacteria, including the antibiotic resistant ones. The biochemical potential and taxonomic diversity of the microbial community in the treated wastewater undergoes changes throughout the year, reflecting seasonal quality fluctuations of the effluent. Sampling points subjected to wastewater discharge presented consistently higher values of total cell number (TCN), prokaryotic biomass (PB), average cell volume (ACV) and live cells percentage (LD), when compared to reference points not subjected to this impact. Also novel pollutants, such as resistant bacteria, resistance genes and integrons were present in wastewater and in wastewater recipients. Their continuous discharge into the environment with wastewater suggests the need for routine monitoring to assess the spread of antibiotic resistance. The proposed method for testing beta-lactam resistance among coliforms can be used worldwide for this purpose, with minimal need for expertise, reagents or sophisticated equipment.

Nomenclature and abbreviations

Abbreviation	Description
A2O	Anaerobic-anoxic-aerobic treatment method
ACV	Average Cell Volume
AOB	Ammonia Oxidizing Bacteria
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Genes
AS	Activated Sludge
AST	Antimicrobial Susceptibility Testing
BOD	Biochemical Oxygen Demand
CAS	Conventional Activated Sludge
CCA	Canonical Correlation Analysis
CEC	Chemicals of Emerging Concern
CFU	Colony Forming Units
COD	Chemical Oxygen Demand
CTX	Cefotaxime
CTX-R	Cefotaxime-resistant
DAPI	4′,6-diamidino-2-phenylindole
DEFT	Direct Epifluorescent Filter Technique
EC	Electical Conductivity
ECOFF	Epidemiological Cut-Off values
EPA	Environmental Protection Agency
ESBL	Extended Spectrum Beta-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
gc	gene copies
HM	Heavy Metals
LD	Live/Dead
LOD	Limit of detection
MBR	Membrane Bioreactor
MIC	Minimum Inhibitory Concentration
N2O	Nitrous oxide
NGS	Next Generation Sequencing
N-NH4	Ammonium nitrogen
N-NO2	Nitrite nitrogen
N-NO3	Nitrate nitrogen
NOB	Nitrite Oxidizing Bacteria
OTU	Operational Taxonomic Unit
PB	Prokaryotic Biomass
PCA	Principal Component Analysis
PCCP	Personal Care and Cosmetic Products
PCR	Polymerase Chain Reaction
PE	Population Equivalent
P-PO4	Phosphate phosphorus
Q	Discharge
SBR	Sequencing Batch Reactor

TCN	Total (Prokaryote) Cell Number
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorus
TSS	Total Suspended Solid
WHO	World Health Organisation
WWTP	Wastewater Treatment Plant

1 INTRODUCTION

From the historical point of view, wastewater treatment and wastewater treatment plants (WWTPs) have greatly improved water quality globally – both in terms of environmental and human health protection (Prasse et al., 2015). Biological treatment methods, typically based on activated sludge (AS) processes, have become the most popular technology applied worldwide, despite its relatively short, 100-years of history (Ardern and Lockett, 1914).

However, municipal wastewater is a complex matrix due to the origin from households, various types of industries, hospitals or stormwater drainage. Apart form fecal matter it contains heavy metals, oil, grease, as well as microplastics and micropollutants, e.g.: pharmaceuticals and personal care products, identified as contaminants of emerging concern (CECs). Even if conventional WWTPs can provide high removal efficiency (>90%) of nutrients and fecal bacteria (Lucena et al., 2004), they are not adapted to eliminate the release of these novel pollutants into the environment (Prasse et al., 2015; Schwarzenbach et al., 2006). CECs are entering the sewage systems as a consequence of the overall economic development, but also changes in the society or human behaviour, e.g. aging population leads to greater medicines use, a pandemic increased the prevalence of disinfectants, strong toilet cleaners, laundry powders or personal care products (Chirani et al., 2021; Teymoorian et al., 2021). Further societal or lifestyle changes create new and challenging implications for the wastewater sector. For example, only recently wastewater treatment processes started to be associated with dissemination of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB), (Michael et al., 2013; Rizzo et al., 2013). The resistant and potentially pathogenic fraction of the effluent's microbial community may survive in the receiving surface waters, posing environmental and epidemiological risk (Berendonk et al., 2015; Manaia, 2017).

For a long time, the main target of municipal wastewater treatment processes were the substances contributing to eutrophication of surface waters (nitrogen, phosphorus and organic compounds). This resulted in a focus on the microbial community of the activated sludge, which was studied in order to understand the functions of individual microorganisms and to increase the efficiency of the wastewater treatment. At the same time, the quality of discharged effluent was defined by chemical constituents, while the microbial community composition of the effluent, its dynamics, fluctuations, quantity or subsequent fate and role in the receiving waters remained largely unknown.

In the context of all the above, determining chemical and microbial composition of the wastewater seems to be particularly important. Due to its complexity, the need for a multidisciplinary strategy to assess its overall quality has been already mentioned in the literature (Prasse et al., 2015). Moreover, treated wastewater and wastewater treatment plants require a deep and holistic approach, based not only on the

effluent quality, but also taking into account its impact on the environment. Considering the constant global population growth, the amount of generated wastewater is going to increase. The anthropogenic-related pressure is also expanding in sensitive areas, such as European Arctic – both due to the growing number of inhabitants or tourists, and the ongoing climate change. Additionally, small human settlements, prevailing in the polar areas, can often discharge untreated wastewater into the environment or tend to be overlooked in wastewater quality or monitoring guidelines, despite being located in a relatively pristine ecosystem.

As mentioned before, the treatment methods, but also the existing legislation on wastewater discharge does not yet follow the new emerging threats or microbial quality of the treated effluent, including e.g. presence of antibiotic resistance indicators. The proposal for a revised Urban Wastewater Treatment Directive suggests new areas for development of wastewater sector, e.g. mitigation of discharge of emerging pollutants, fecal indicators, as well as by recognizing the risks associated with currently overlooked, small (below 2 000 PE) agglomerations. To address the novel risks, the appropriate and informative monitoring should be conducted. Thus, the development of universal, standardized procedures for global surveillance is needed. In the context of all the above, this thesis aimed to (1) fill in the knowledge gaps regarding the wastewater composition and its impact on the recipient, (2) identify the microbial parameters important for wastewater quality assessment and (3) propose and test a widely applicable, inexpensive tool to monitor the recognized threat associated with discharge of antibiotic-resistant coliforms.

2 OBJECTIVE AND SCOPE

The main aims of this thesis were to:

1) characterize the wastewater composition by means of a wide range of chemical and microbiological parameters, benefiting from the synergistic advantage of mutually complementary methods,

2) investigate the impact of the wastewater discharged to the surface waters in different geographical locations, using chosen chemical and microbial parameters,

3) develop a universal, informative, low-cost and easy-to-use method to monitor the environmental impact of wastewater discharge in terms of antibiotic-resistant coliforms discharge, recognized as a global issue of concern.

In order to fulfil the main aims, the **secondary objectives** of the thesis were defined, as specified in the **Table 2**. In order to achieve the objectives, the corresponding tasks were defined as the **scope of the research** (**Table 2**) and addressed using a synergistic approach, what is described further in **Section 4 - Methodology**.

Table 2 Thesis objectives and scope of the research

	Thesis objectives	Corresponding scope of research	Manuscript
Chemical parameters	Characterize the chemical profile of the wastewater and the recipient	Investigate the chemical parameters of wastewater: a. Heavy metals (Cd, Cr, Mn, Fe, Co, Ni, Cu, Zn, Hg, As, Pb, V, U) b. Nutrients (N and P compounds, TOC, COD, BOD) c. Other physicochemical parameters (TSS, pH, EC)	I, II, III
Cell number and viability	Quantify the bacterial load discharged with treated wastewater to the recipient	a. Visualise the complex microbial community b. Estimate the abundance and biomass of prokaryotic cells in treated wastewater and the recipient c. Quantify the active share of the microbial community	II, III
Microbial community composition	Analyse to what extent the microbial community of the treated wastewater reflects the taxonomic structure of the recipient	a. Identify the taxonomic structure and investigate temporal variations in the microbial community of the treated wastewater b. Compare the microbial community composition and presence of selected bacterial groups in the treated wastewater and its recipient, taking into account the reference points not subjected to direct WWTP effluent discharge	II, III
Biochemical potential	Recognize the potential functions of the microbial community of the WWTP effluent and the recipient	a. Determine the relative abundance of functional bacterial groups: nitrifiers (ammonia- and nitrite-oxidizing bacteria) and denitrifiers in various samples b. Quantify the N-cycling genes present in the microbial community	II, III
Sanitary aspect	Investigate the sanitary status of the wastewater and recipient	a. Quantify chosen fecal indicators b. Confirm the taxonomic assignment by advanced biochemical methods	II, III, IV
Antibiotic resistance	Explore the antibiotic resistance of fecal indicators in the wastewater and the recipient	a. Assess the antibiotic resistance profile of *Enterococcus* spp. b. Detect presumptive extended-spectrum beta-lactamase producing bacteria via quantification of cefotaxime-resistant fecal coliforms	II, IV

3 THESIS CONCEPT AND STRUCTURE

Thesis is structured based on the four appended papers, according to **Figure 1**. **Manuscripts I and II** present case studies in the polar areas, particularly vulnerable to human-induced changes. **Manuscript III** represents large WWTPs in temperate climate on the example of two units located on Polish coast, whereas **Manuscript IV** is a global study (**Table 3**). Various localizations have been chosen due to universality of the topic and its significance all over the world. Sequence of Manuscripts reflects the differences between case studies: the geographical location, applied treatment methods and the gradient of wastewater treatment development stages: from no treatment at all (**Manuscript I**), through small, domestic-like scale (**Manuscript II**), up to full scale WWTPs (**Manuscript III and IV**). In order to assess the WWTP effluent impact, results were compared with the reference points not subjected to the wastewater discharge.

Order of Manuscripts appended in this thesis corresponds also to the investigation of chemical parameters and further increasingly advanced and detailed study of the microbial parameters in the wastewater and environmental samples. The synergistic approach, combining various research methods has been used to take advantage of the strengths of different analytical techniques and minimize their weaknesses, in order to get possibly the fullest picture of the wastewater discharge influence on the receiving water bodies.

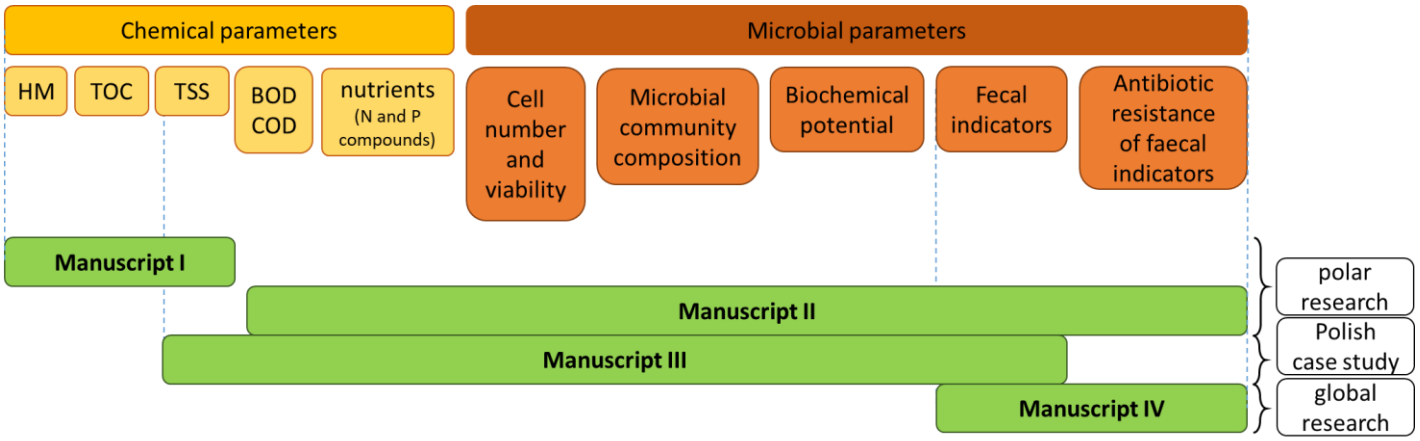

Figure 1 The schematic overview of the main parameters tested in the scope of the PhD thesis.

Table 3 Characteristic of the sampling area of each Manuscript

Manuscript	**Location**	**WWTP**	**Treatment technology**	**Inhabitants served**	**Average discharge**	**Wastewater recipient**	**Climate zone**
I - Heavy Metals in a High Arctic Fiord and Their Introduction with the Wastewater: A Case Study of Adventfiorden-Longyearbyen System, Svalbard.	Longyearbyen, Svalbard, European Arctic	none	none	2 500	780 m^3/d (submarine pipe)	Fiord – marine waters	Polar
II - The microbial community, its biochemical potential, and the antimicrobial resistance of Enterococcus spp. in Arctic lakes under natural and anthropogenic impact	Polish Polar Station Hornsund, Svalbard, European Arctic	small scale	Mechanical and biological (nitrification/denitrification system in SBR)	10 – 46	0.6 – 3.6 m^3/d (surface discharge)	Lake	Polar
III - Insights into the microbial community of treated wastewater, its year-round variability and impact on the receiver, using cultivation, microscopy and amplicon-based methods	Gdańsk and Gdynia agglomerations, Poland	2 full scale WWTPs	Mechanical and biological (A2/O or Bardenpho system) with advanced nutrient removal, and optional chemical precipitation (for phosphorus removal)	570 000 and 360 000	92 958 and 55 294 m^3/d (submarine pipe)	Coastal marine waters	Temperate
IV - A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants	Global: 5 continents, 22 countries	57 full scale WWTPs	Various: mechanical-biological (trickling filters / CAS / MBR), with occasional disinfection (chlorination/ UV/ membrane filtration	500 – 1 400 000	700 – 2 100 000 m^3/d (various, unspecified)	Various, not specified	Various: temperate, subtropical and tropical

4 METHODOLOGY

This thesis combines the results of various, mutually complementary chemical and microbial analysis, which are summarized in **Figure 1** and presented in **Table 4**. The details of each method can be found in the corresponding appended Manuscripts. The application of wide range of analysis was motivated by the need of holistic view on the subject of wastewater discharge and impact on the receiving waters. Combining various analytical techniques provides a synergistic benefit enabling to leverage strengths of different research methods while minimizing their weaknesses. Additionally, WWTP effluent was analysed together along with its receiving bodies and reference points (isolated from direct anthropogenic, wastewater-related influence). This aimed to better represent the environmental changes induced by wastewater discharge itself.

Table 4 Summary of the analytical methods applied in the dissertation

	Parameter	**Shortcut**	**Unit**	**Method / Equipment**	**Manuscript**
Chemical parameters	Heavy metals (Cr, Mn, Fe, Co, Ni, Cu, Zn, Hg, As, Cd, Pb, U, V)	HM	µg/L	Inductively Coupled Plasma Mass Spectrometry, Thermo XSERIES 2 ICP-MS (APHA 1985)	I
	Total Suspended Solids	TSS	mg/L	membrane filtration method; APHA 2005	I, II, III
	Total Organic Carbon	TOC	mg/L	Schimazu TOC-VCSH/CSN Analyser; APHA 2005	I
	pH	-	[-]	WTW pH/Oxi 3401; APHA 2005	II
	Electrical conductivity	EC	µS/cm		II
	Nutrients (TN, N-NO_2, N-NO_3, N-NH_4, TP, P-PO_4)	-	mg/L	XION 500 Spectrophotometer; APHA 2005	II, III
	Chemical Oxygen Demand	COD	mg O_2/L		
	Biological Oxygen Demand	BOD	mg O_2/L	manometric respirometric BOD OxiTop® method; APHA 2005	III
Microbial parameters	Total (prokaryotic) Cell Number	TCN	cells/mL	Direct Epifluorescent Filter Technique (DEFT); 4′,6-diamidino-2-phenylindole (DAPI) staining	II, III
	Average Cell Volume	ACV	µm^3		
	Prokaryotic Biomass	PB	µg C/dm^3		
	Cells viability (percentage of live cells)	LD	%	Direct Epifluorescent Filter Technique (DEFT); membrane integrity-based viability assay (Live/Dead assay)	II, III

Microbial parameters	Microbial community composition	-	Relative abundance [%]	Next Generation Sequencing, Illumina	II, III
	N-cycling genes quantification	-	gc = gene copies gc/mL sample, gc/16S rDNA, gc/µL DNA, gc/ng DNA	Quantitative real time polymerase chain reaction (qPCR)	III
	Abundance of chosen sanitary indicators: *Enterococcus*, *Enterobacteriaceae*, coliforms, fecal coliforms, *E. coli*	-	CFU (colony forming units)/100 mL	Cultivation on selective media according to PN-EN ISO 7899-1, PN-EN ISO 7899-2 (*Enterococcus*), PN-EN ISO 9308-3, PN-EN ISO 9308-1 (*coliforms, fecal coliforms, E. coli*), PN-EN ISO 21528-2:2017-08 (*Enterobacteriaceae*)	II, III, IV
	Biochemical strain identification	ID	percentage of agreement with database (Snyder et al., 2008)	Identification based on strain biochemical response to a set of fluorogenic and chromogenic substrates; automated bacterial identification and susceptibility testing system BD Phoenix™	II
	DNA-based strain identification			16S rRNA gene sequence analysis / MALDI-TOF MS	IV
	Antimicrobial susceptibility testing	AST	mg/L - MIC system (according to EUCAST)	Detection of organism growth on broth-based microdilution test; automated bacterial identification and susceptibility testing system BD Phoenix™ (classification to susceptible/resistant according to EUCAST MIC guidelines)	II
			Growth or lack of growth	Cultivation on selective media with given concentration of antibiotic (cefotaxime, 4 µg/mL), according to EUCAST guidelines	IV

5 RESULTS AND DISCUSSION

5.1 MANUSCRIPT I - HEAVY METALS IN A HIGH ARCTIC FIORD AND THEIR INTRODUCTION WITH THE WASTEWATER: A CASE STUDY OF ADVENTFIORDEN-LONGYEARBYEN SYSTEM, SVALBARD

Manuscript I presents the topic of untreated wastewater discharge in polar areas on the example of Longyearbyen, the capital of Svalbard archipelago and the biggest among the few human settlements on the Spitsbergen island. Longyearbyen has experienced rapid growth over the past century (from 25 people in 1906 to more than 2,500 permanent residents in 2021, with almost doubling the population over last two decades, Statistics Norway 2022). Furthermore, the tourist traffic in the polar areas is gradually increasing. Number of tourists visiting Longyearbyen during the year reached almost 170,000 in 2019 (MOSJ, 2019), the number of people arriving by plane doubled over the last ten years and the larger cruise ships coming to the city host more passengers and crew on board than there are Longyearbyen residents (AidaLuna 2020). Thus, a massive tourist business operates in the town. Multiple rental companies offer as many snowmobiles as there are permanent inhabitants, and all the supplies are transported by sea or air. All that lead to increasing human impact, including especially fuel-related emissions, but also larger surface water usage for drinking and household purposes, and consequently growing wastewater generation.

The wastewater in Longyearbyen is mostly of the domestic origin, the industry is limited mainly to accommodation, food service, construction, administration and education, with smaller shares of wholesale and retail trade, repair of motor vehicles and motorcycles, transportation and storage, as well as mining and quarrying (Statistics Norway 2020). Due to location on the island, all waste has to be transported by ship to the mainland and organic waste cannot be landfilled or collected. Therefore, sinks in Longyearbyen are equipped with grinders for food scraps, what affects the amount of organic waste disposed to the fiord with the untreated wastewater. Currently the city of Longyearbyen discharges untreated wastewater by a submarine collector to the nearby fiord (Adventfiorden), where mixing and dispersion is provided by the geographical location and natural phenomena: estuaries of two glacial rivers, ocean tides and currents ensuring water circulation.

Until now, local research on the wastewater discharge were focused on modelling the wastewater-related contaminants dispersion, based on hydraulic and hydrology studies. The environmental impact of nutrients and heavy metals was studied in terms of their influence on benthic fauna and sediments (Akvaplan-niva Rapport 2010-2020). However, recent studies suggest that heavy metals can be also important from the perspective of growing antimicrobial resistance among bacteria, as they might promote the spread of antibiotic resistance via co-selection (Seiler and Berendonk, 2012).

Therefore, in **Manuscript I** the concentrations of heavy metals (HMs: Cd, Cr, Mn, Fe, Co, Ni, Cu, Zn, Hg, As, Pb, V, U), were selected as an indicator of anthropogenic impact associated with untreated wastewater discharge to the environment. Together with total suspended solids (TSS) and total organic carbon (TOC), these parameters were analysed during two sampling campaigns (July and October) in wastewater and in the fiord being the wastewater recipient. Spatial distribution of heavy metals in the waters of the recipient has also been investigated, taking into account the tides, ocean currents, depth and distance from the wastewater discharge (for details see **Fig. 1 in Manuscript I**). **Figure 2** presents the schematic overview of the parameters tested in scope of **Manuscript I**. According to the best knowledge, this is one of the few studies concerning heavy metals concentration in the seawater Svalbard's fiords and the first one on Adventfiorden (Zaborska et al., 2020).

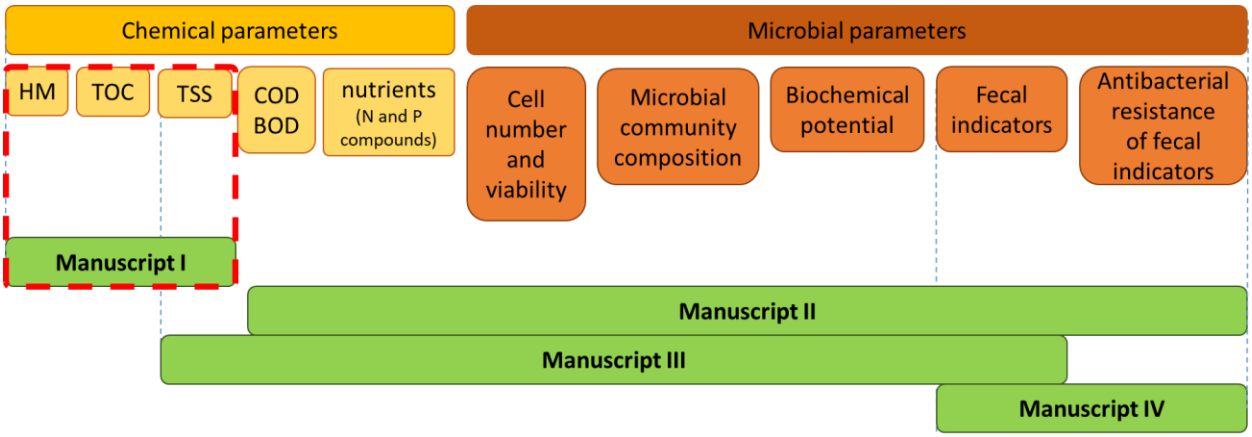

Figure 2 Schematic overview of the parameters tested in Manuscript I, marked with a red frame.

5.1.1 Chemical parameters of the wastewater and the recipient

The results of **Manuscript I** show that the concentrations of heavy metals in untreated wastewater generated in Longyearbyen are similar or lower than typical untreated European domestic wastewater or wastewater generated by polar scientific stations (**Table 5**). Yet, they are higher than the levels in their recipient, Adventfiorden, therefore wastewater can be considered as a heavy metal source for the aquatic environment of the wastewater receiver.

Concentrations of heavy metals in the fiord generally fall into the range typically met in the marine waters (**Table 3 in Manuscript I**). Their sources in the environment can be attributed to current human activities (fossil fuel combustion: power plant operation, intensive and increasing tourism - air, automobile and snowmobile traffic, marine deliveries) but also to the geology and mining history of the studied area. It is worth to note that increased concentrations of some heavy metals, especially iron and manganese were indeed detected in the surface water reservoirs serving as a source of tap water for the town (up to 1650 and 660 µg/L, respectively), as well as in the potable water system itself (up to 98 and 510 µg/L, respectively, **Table 5**, **Table 3 in Manuscript I**).

It is important to note that the fiord being the wastewater recipient receives a lot of sediments, mostly inorganic, discharged by two glacial rivers, while wastewater-related solids correspond mainly to organic compounds. Due to waste management system in the town, wastewater contains not only faeces, but also food scraps from households and restaurants, what increases the organic load. Thus, in **Manuscript I**, two parameters were proposed in order to distinguish the anthropogenic from natural impact: total suspended solids (TSS) was mostly in mineral form and represented mainly the riverine inflow, while total organic carbon (TOC) was supposed to reflect rather the wastewater discharge. Indeed, TOC was on average 40 times higher in wastewater than in the recipient. In fiord, TOC showed minor fluctuations (from 0.5 to 1.4 mg/L), with maximum values at sampling points in the vicinity of wastewater collector outlet and at the mouth of two rivers (for details see **Fig. 3 in Manuscript I**). The influence of wastewater discharge, vegetation or animal activity on the obtained results can be speculated, but no unambiguous conclusions can be drawn based on the limited amount of data. On the contrary, TSS clearly reflected the river inflow carrying mineral sediments, with maximum value at the estuary (835 mg/L) and decreasing both spatially and seasonally with reduced river impact (lower values further from the mouth or in colder months when water flow ceases).

Table 5 Concentrations of heavy metals in certain sample types, expressed as min-max values (the literature data are shown as the lowest and highest values among those presented in corresponding papers). Table adapted from Table 3 and Table 4 from Manuscript I.

Sample type	**Concentration (µg/L)**											**Reference**
	As	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mn**	**Ni**	**Pb**	**V**	**Zn**	
Surface reservoirs of tap water, Longyearbyen	0.03-0.25	0.02-1.00	0.05-12.68	0.1-4.3	0.1-9.3	5.0-1650	4.0-660	0.1-30.9	0.04-1.32	<LOD-0.06	0.9-77.4	Longyearbyen Lokalstyre Annual Reports, 2002–2017
Tap water, Longyearbyen	0.03-0.1	0.01-1.0	no data	no data	no data	6.0-98	5.0-510	no data	no data	no data	no data	
Drinking water limits	10	5	-	50	100	200	50	20	10	-	-	EPA 2018
Untreated domestic wastewater, Longyearbyen	1.57-2.55	0.02-0.03	1.55-1.57	1.48-1.51	1.69-2.73	170-282	132-220	11.9-13.1	0.74-1.49	1.47-2.61	3.9-12.3	**Kalinowska et al. 2020, Manuscript I**
Untreated wastewater from polar scientific stations	no data	0.45-4.2	1.68-3.33	4.5-32	4.3-870	428-1646	13-176	23.3-42	0.48-376	no data	38-1210	Crockett, 1997; Stark et al., 2015; Szopińska et al., 2021
Untreated European domestic wastewater	0.5-0.6	0.1-1	no data	1-4	7-78	45-963	33-77	3-6.2	2-5.5	no data	36-181	Lesage et al., 2007; Moriyama et al., 1988; Thévenot et al., 2007
Untreated European municipal wastewater	1.4-4	0.5-21	no data	13-40	35-125	480-4785	67-452	13-770	16-63	6	230-470	Chipasa, 2003; Karvelas et al., 2003; Thévenot et al., 2007, Gdansk-Wschód - WWTP Reports - Unpublished data (2013–2014)
Treated European domestic wastewater	no data	0.10	no data	1.00	3.00	28	227	3.00	2.00	no data	26.0	Lesage et al., 2007
Wastewater recipient, Longyearbyen	0.42-2.01	0.01-0.04	0.02-0.32	0.1-0.4	0.15-1.58	0.94-4.86	0.14-3.88	0.06-1.01	0.09-0.13	0.25-0.91	0.56-3.35	**Kalinowska et al. 2020, Manuscript I**

5.1.2 Conclusions

The results of **Manuscript I** show that the concentrations of heavy metals in the untreated wastewater from Longyearbyen are relatively low and to large extend mirror the concentrations met in the tap water, which is obtained from the surface reservoirs (Hodson et al., 2016). Therefore, the advanced treatment for their removal would be neither necessary nor rational in this case. Methods for heavy metals removal are continuously developed, however they are mostly applied for heavily polluted industrial wastewater (Chipasa, 2003; Shrestha et al., 2021) and are of high operational costs due to the chemicals used, high-energy consumption and handling costs for toxic sludge disposal (Gunatilake, 2015).

Based on the literature studies, the anthropogenic sources of heavy metals in the wastewater from Longyearbyen include cosmetics, laundry detergents, medicines, plumbing, food residues and faeces (**Table 1 in Manuscript I**). Thus, it seems that major reduction of wastewater-related emissions could be achieved through an adequate management and regulations. The settlement is located on an island and all goods must be delivered from the mainland, therefore, the usage of eco-friendly cosmetics, detergents and cleaning agents could possibly be encouraged or even enforced e.g. by introducing products recommendations or a list of prohibited ingredients. The majority of groceries, cosmetics and other everyday products are supplied by one supermarket, which makes the control of the articles easier. At this moment, reducing heavy metals emissions by influencing customer choices and habits may be a more economically rational approach for this settlement than investing in advanced treatment methods, especially taking into account strong link to energy consumption and the need to adapt to local climatic conditions.

Nevertheless, the growing number of residents and tourists in the Arctic is increasing wastewater discharge, which contributes significantly to the human-induced impact. Even if the concentrations of heavy metals in the wastewater does not seem to be particularly high, there are other substances of anthropogenic origin and special concern that should be taken into account in pollution monitoring and environmental impact assessment, e.g. personal care and cosmetic products (PCCPs), such as microplastics or antibiotics. Additionally, the presence of pharmaceuticals and their residues in wastewater (Szopińska et al., 2021) can create a selective pressure for antibiotic resistance dissemination among microorganisms, and heavy metals may additionally promote its spreading through co-selection (Seiler and Berendonk, 2012).

The discharged wastewater also introduces to the fiord an increased amount of allochthonous bacteria of human gut origin and organic waste (faeces, food scraps) that can support their survival. Indeed, the microscopic analysis of samples from Longyearbyen has shown that untreated wastewater contained 2-3 orders of magnitude more bacteria than the waters of the recipient (Kalinowska et al. unpublished data). This preliminary study has also revealed the presence of resistance genes to sulphonamides and integrons in the Longyearbyen's wastewater and its recipient (Kalinowska et al., unpublished data). Integrons are

genetic elements that can also be involved in spreading the antibiotic resistance among bacteria (Stalder et al., 2012) and their abundance in the untreated wastewater was found to be even two magnitudes higher than the receiving fiord waters. This raises the question of the associated risk to human health, as well as the necessity of the wastewater treatment taking into account the sanitary and epidemiological point of view. In this context, the lack of baseline conditions, necessary to evaluate the environmental changes caused by human activity, is particularly alarming.

All the above findings lead to another study of Arctic environment, presented in **Manuscript II**, where combining the chemical and microbial parameters aims to provide a holistic overview on wastewater discharge. Merging various analytical methods offers the advantage of synergy, reducing the uncertainties and weaknesses of each individual assay and allowing the results to be mutually confirmed. For this reason, the synergistic approach was applied in **Manuscript II**, combining chemical, microscopic, cultivation and DNA-based methods.

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"Influence of wastewater on the bacterioplankton community and its characteristics in Adventfiorden, Svalbard", Arctic Field Grant no 282607, funded by Research Council of Norway, Svalbard Science Forum

The above-discussed results were presented during following conferences:

1. **Kalinowska A.,** Szopińska M. , Chmiel S. , Kończak M., Polkowska Ż. , Jankowska K. , Artichowicz W, Łuczkiewicz A., *"Fate of heavy metals in the Arctic fiord - is the untreated wastewater discharge a major threat?"*, 2nd IWA Polish YWP Conference, 12-14.02.2020, Warszawa

2. **Kalinowska A.,** Szopińska M. , Chmiel S. , Kończak M., Polkowska Ż. , Jankowska K. , Artichowicz W, Łuczkiewicz A. „*Wastewater discharge as an important activity in the Arctic coastal waters - case study of Longyearbyen, Spitsbergen",* 4th Coastal, Offshore & Ocean Engineering Conference, 15.11.2019, Gdańsk

3. **Kalinowska A.,** Szopińska M., Chmiel S., Kończak M., Polkowska Ż., Jankowska K., Artichowicz W., Łuczkiewicz A., "*Zrzut ścieków nieoczyszczonych a jakość biochemiczna odbiornika w rejonie Longyearbyen, Spitsbergen"* II Konferencja Naukowa Polskich Badaczy Morza, 24-25.09.2019, Gdynia

4. **Kalinowska A**., Szopińska M. , Chmiel S. , Kończak M., Polkowska Ż. , Jankowska K. , Artichowicz W, Łuczkiewicz A., „Status quo of the arctic wastewater recipient – environment modification by chemical compounds including selected heavy metals (Longyearbyen, Svalbard)", 15th International Students Conference Modern Analytical Chemistry, 19-20.09.2019, Praga

5.2 MANUSCRIPT II - THE MICROBIAL COMMUNITY, ITS BIOCHEMICAL POTENTIAL, AND THE ANTIMICROBIAL RESISTANCE OF ENTEROCOCCUS SPP. IN ARCTIC LAKES UNDER NATURAL AND ANTHROPOGENIC IMPACT (WEST SPITSBERGEN)

Manuscript II presents the results of treated wastewater discharge into the polar areas on the example of a small, 10-40 people settlement, Polish Polar Station in Hornsund. The station has been in operation since 1957, what results in relatively small ($Q \leq 3.6$ m^3/d), but continuous anthropogenic emissions. It is located in South Spitsbergen National Park, therefore in order to minimize the human impact, domestic wastewater is treated in small-scale WWTP. Biological fixed-film system (rotating biological contactor) has been installed in 1985 and changed to mechanical-biological system (sequencing batch reactors - SBR) in 2001. Minor modernization was done in 2016 (Wilk and Cimochowicz-Ribicka, 2018).

In **Manuscript II** treated wastewater was sampled together with two nearby small oligotrophic lakes. Lake 1 is a small shallow basin (130 m long, 40 m wide and 1.5 m deep) of glacial origin. It is a source of tap water for the Station and remains only under natural (animal) impact. Lake 2 is slightly smaller and shallower (1 m deep) basin located on moss tundra and stays under anthropogenic impact, receiving the effluent from the Station's WWTP. The impact on both lakes may be connected with supply of nutrients or fecal bacteria of animal or human origin, but in context of wastewater impact, Lake 1 was assumed to serve as the reference, as it reflects the natural state of Arctic tundra lake. In order to assess the importance of animal influence, Lake 1 was sampled at two locations: (1) at the inflow of the tundra stream (point L-TS) passing through bird colonies and (2) point of tap water withdraw (point L-WS – water supply), partially isolated from inflow of suspended solids with a bottomless barrel. However, in the context of this dissertation, the main focus will stay on the human-related impact associated with wastewater disposal.

In the scope of **Manuscript II**, the chemical and microbiological characteristics of WWTP effluent (WW-E), wastewater recipient (WW-R) and reference points (L-TS, L-WS) were analyzed (**Figure 3**). Microbial community composition, quantity and biochemical potential were investigated together with their sanitary status. Special attention was given to chosen fecal indicators, *Enterococcus* spp. and their antibiotic resistance, as polar areas are still considered one of the most pristine environments of limited anthropogenic impact, while WWTPs just recently have been recognized as hotspots for resistance dissemination.

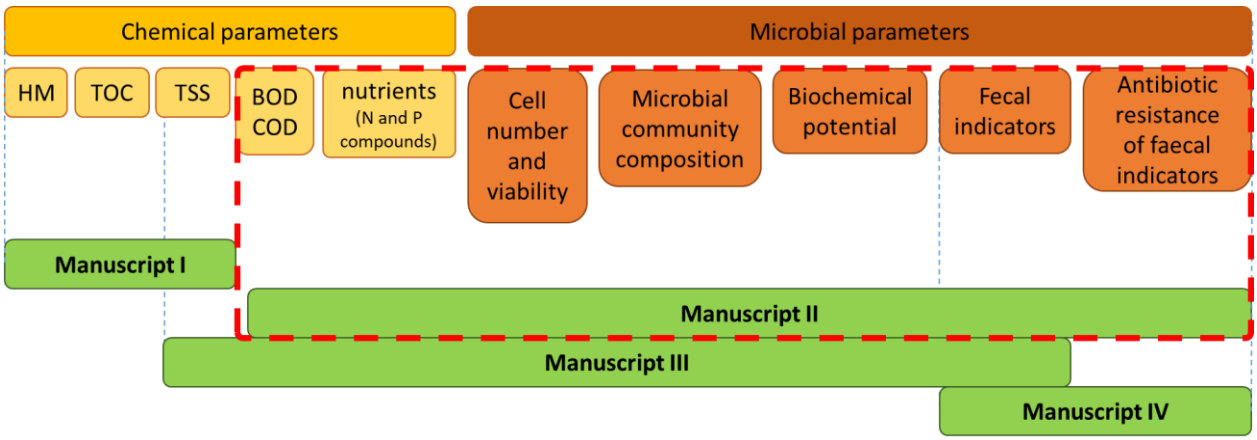

Figure 3 Schematic overview of the parameters tested in Manuscript II

5.2.1 Chemical parameters

Chemical analysis presented in **Manuscript II** included standard parameters required by Polish legislation (Dz.U. 2019 poz. 1311) to monitor the quality of treated wastewater (chemical oxygen demand COD, total nitrogen TN and total phosphorus TP). Concentrations of nutrients and COD in treated wastewater from Polish Polar Station were relatively high (**Table 6**) – higher than typical values of WWTP effluents (Gonzalez-Martinez et al., 2018; Pereao et al., 2021; Szklarek et al., 2021). This is however typical for small polar settlements (Daley et al., 2015; Stark et al., 2016), where water use is limited by supply challenges, resulting in concentrated outflow (Connor, 2008). Additionally, small WWTPs working for only few residents are especially difficult to operate due to the fluctuous inflow and load leading to poor system stability. Despite good reduction efficiency of the WWTP (Table 6, compared with other small-scale WWTPs (Abegglen et al., 2008), parameters such as COD, TN and TP temporarily exceeded the limit values defined by Polish and European regulations (Dz.U. 2019 poz. 1311, Council Directive 91/271/EEC). It is particularly significant given the extremely sensitive environment into which this wastewater is discharged.

All of the tested parameters (electrical conductivity, COD, nitrogen and phosphorus compounds) were 1-2 magnitudes higher in the treated wastewater than in the studied lakes. In Lake 1, the sampling point L-TS corresponded to inflow of the tundra stream passing through the bird colonies area, while L-WS represents the point of water supply (point of tap water uptake for the Station), isolated from direct impact of surface run-off, animal feces or surrounding tundra vegetation. At L-TS slightly higher values were recorded than at L-WS, what indicates the role of natural influence on shaping the chemistry of Arctic lakes.

Lake 2 being the wastewater recipient, presented nutrient concentrations of at least 2 times greater than in Lake 1 that remains only under natural influence (**Table 6**). This indicates the significant impact of the wastewater discharge on the small oligotrophic lake, which has been confirmed by the results of microbial analysis, discussed further.

Table 6 Chemical characteristics of two Arctic lakes and wastewater studied in Manuscript II. Data shown as range. EC – electrical conductivity, COD – chemical oxygen demand, LOD – level of detection. Bold font indicates results exceeding permissible values.

Parameter	Unit	Lake1 – under natural impact		Lake2 – under human impact	Raw wastewater	Treated wastewater	Polish requirements for treated wastewater discharge[1]	European requirements for treated wastewater discharge[2]		Average reduction for Polish Polar Station WWTP[4]
		L-TS	L-WS	WW-R	–	WW-E		mg/L	min. % reduction	
EC	µS/cm	143 – 154	122 – 137	171 – 211	–	1010 – 1115	–	–	–	–
COD	mg/L	<5	<5	24.7 – 35.3	>12 000 (>LOD)	**147 – 189**	**≤150**	**≤125**	75	99%
TN		0.35 – 1.75	<0.1 (<LOD)	1.54 – 2.47	357	**62.4 – 80.8**	**≤30**	**≤15**[3]	70-80	76%
N-NH_4		0.10 – 0.76	0.04 – 0.2	0.79 – 1.51	80.7	29.1 – 39.8	–	–	–	58%
N-NO_3		<0.25 (<LOD) – 0.40	<0.25 (<LOD)	0.66 – 1.05	1.2	4.5 – 8.8	–	–	–	–
TP		<LOD (<0.05)	<LOD (<0.05)	0.16 – 0.34	93.3	**7.0 – 10.8**	**≤5**	**≤2**[3]	80	90%
P-PO_4		<LOD (<0.05)	<LOD (<0.05)	0.10 – 0.28	39.2	5.4 – 9.4	–	–	–	81%

[1] Requirements for WWTPs below 2000 people equivalent (PE), according to Polish standards (Dz. U 2019 poz 1311);

[2] Requirements for discharges from urban waste water treatment plants (The Urban Waste Water Treatment Directive 91/271/EWG)

[3] Requirements for discharges from urban waste water treatment plants. Concentrations of nitrogen and phosphorus are defined for WWTPs above 10 000 PE discharging effluent to sensitive areas which are subject to eutrophication. - one or both parameters may be applied depending on the local situation. The values for concentration or for the percentage of reduction shall apply.

[4] the minimal percentage reduction of compounds is specified only for WWTPs larger than 2000 PE (Dz. U 2019 poz 1311)

5.2.2 Quantification of the prokaryotic community and its viability

Quantification of the microbial community and its viability has been done using the direct epifluorescent technique (DEFT) and application of appropriate staining substances. Propidium iodide and SYTO9 were applied for membrane integrity-based viability assay (so called Live/Dead assay), providing the percentage of alive cells in the total community (LD), while 4′,6-diamidino-2-phenylindole (DAPI) was applied for determination of total prokaryotic cell number (TCN), prokaryotic biomass (PB) and average cell volume (ACV). These parameters define the state of the microbial community, but can also correspond to the environmental conditions it lives in: e.g. nutrient availability or temperature (Danovaro and Fabiano, 1997; La Ferla et al., 2010; La Ferla et al., 2014).

Among all the samples described in **Manuscript II**, the WWTP effluent (WW-E) presented the highest average values of cell number (4.6×10^6 cells/mL), prokaryotic biomass (137 µg C/L) and live cells (16%), matching the favourable conditions for bacteria development found in the wastewater. The average cell volume was characterized by very small scatter (**Table 7**, **Figure 2 in Manuscript II**). Total prokaryotic cell number (TCN) and prokaryotic biomass (PB) in the treated wastewater were on average 2-4 times higher than in Lake1 and Lake2, but also higher than values typically met in large WWTPs' effluents (e.g. please compare with **Manuscript III**).

All the results of microscopic analysis were lower for Lake1 remaining under natural (animal) impact than for Lake2 being the wastewater recipient (**Table 7, Figure 2 in Manuscript II**). Small differences between sampling points in Lake1 were attributed to slightly higher nutrient concentrations at tundra stream inflow (point L-TS), possibly introducing some substances from nearby bird colonies. Higher microbial results in Lake 2 were also in line with higher levels of nutrients in this reservoir and corresponded to the discharge of treated wastewater. Compared with literature data, Lake 1 and Lake 2 showed similar, but slightly higher values than other surface waters from the nearby area (Górniak et al., 2016; Kosek et al., 2019, 2018). This may result from the characteristics of the tested reservoirs: they receive nutrients of natural and anthropogenic origin and are more prone to warming up due to their shallow, stagnant character, what can create a more favourable niche for bacteria survival and development.

Table 7 Results of microscopic analysis in comparison to the literature data from the area. TCN – total (prokaryotic) cell number, PB – prokaryotic biomass, LD - live cells expressed as a percentage of the total community, nd – not determined

Sample type	Sample name	TCN [$\times10^6$ cells/mL]	PB [µg C/L]	ACV [µm^3]	LD [%]
Lake1 – under natural impact	L-TS	0.98-1.44	27-31	0.08-0.15	8.0-8.3
	L-WS	0.94-1.38	22-27	0.07-0.09	5.6-6.4
Treated wastewater	WW-E	4.04-5.07	116-153	0.13-0.14	14.9-16.1
Lake2 – under anthropogenic impact	WW-R	2.10-2.52	54-69	0.10-0.20	9.5-11.4

5.2.3 Microbial community composition

Treated wastewater was dominated by 10 phyla (**Fig. 4c in Manuscript II**), constituting for 96% of its community, each of them reaching the relative abundance from 3% to 21%. They included *Actinobacteria, Proteobacteria* (21% each), *Dojkabacteria, Chloroflexi, Planctomycetes* (10-14%), with smaller shares of *Bacteroidetes, Firmicutes, Parcubacteria, Microgenomates* and *Saccharibacteria* (3-6%). These results reflect taxa dominating activated sludge reactors (Saunders et al., 2016; Zhang et al., 2017; Zheng et al., 2016), also those located in the Arctic (Gonzalez-Martinez et al., 2018).

Lake 2, being the wastewater recipient, presented the highest microbial diversity among all of the tested samples (**Figure 3d in Manuscript II**), what reflects mixing of natural autochthonous and WWTP-related allochthonous bacterial community. Taxonomic composition of Lake 2 highly mirrored the taxa found in treated wastewater (**Figure 4**), containing analogically higher shares of *Sachharibacteria/TM7*, *Dojkabacteria/WS6*, *Microgenomates*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, *Firmicutes* than Lake 1, not impacted by the wastewater. Similarities between treated wastewater and its recipient were also visible at lower taxonomic levels and reflected by the presence of bacteria associated with activated sludge or human gut (eg. genus *Phenylobacterium*, families *Isospheraceae, Pirellulaceae, Nocardioidaceae, Anerolinaceae*, class *Clostridia*). In total, Lake 2 (sample WW-R) shared 258 operational taxonomic units (OTUs) with treated wastewater (sample WW-E), out of which 165 OTUs were unique to these samples and not found in Lake 1. Higher share of *Cyanobacteria* in Lake 2 than in Lake 1 was associated with an increased nutrient concentrations affiliated with WWTP effluent inflow. All this confirm the impact of the treated wastewater discharge on the waters of its recipient.

Lake 1 exhibited lower microbial diversity than Lake 2 or wastewater, and it was dominated by only three phyla: *Proteobacteria* (*Alpha-* and *Beta-* subdivisions), *Actinobacteria* and *Bacteroidetes*, that constituted for over 93% of the total community. Composition of microbial community in Lake 1 was clearly noticeably different from Lake 2 – it was characteristic to freshwater reservoirs and highly similar to other studies of Arctic surface waters nearby Polish Polar Station (Kosek et al., 2019; Ntougias et al., 2016), which might be shaped to some extent by endophytic population of Arctic tundra (Nissinen et al., 2012) or microbial composition of Arctic soils (Campbell et al., 2010; Neufeld and Mohn, 2005)S. Two sampling points in Lake1 (L-TS and L-WS) were highly similar to each other, with only minor discrepancy for phylum *Cyanobacteria* – their development at point L-TS might have been supported by tundra stream introducing nutrients from bird colonies.

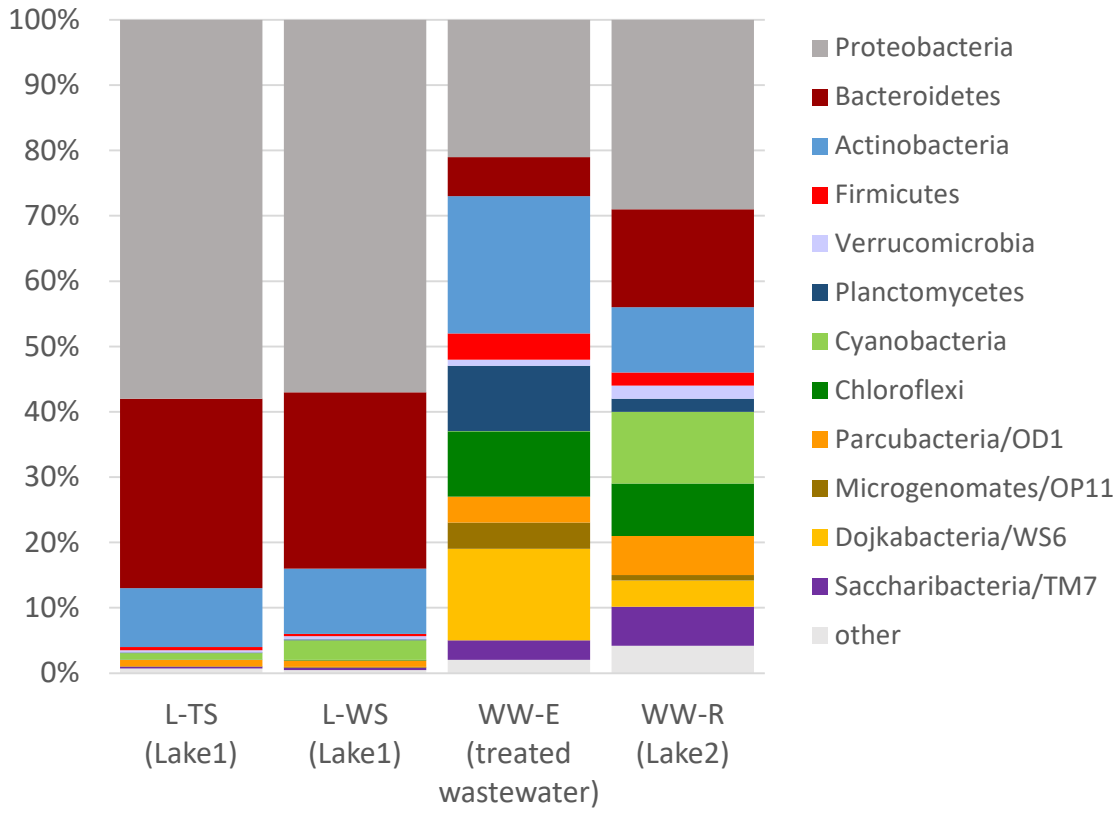

Figure 4 Microbial community composition of the samples analyzed in Manuscript II presented on phylum level

Results of Next Generation Sequencing (NGS) analysis were also consistent with the cultivation method applied to assess the overall sanitary status of the samples (**Section 5.2.4**). NGS confirmed the highest relative abundance of *Enterococcus* spp. in treated wastewater (WW-E, 0.09%), followed by wastewater recipient (WW-R, 0.04%) and Lake1 (L-WS and L-TS, <0.01%). Similar abundance patterns were revealed for other fecal indicators and gut-related taxa: *Escherichia* (0.1%, 0.06% and ≤0.01%, respectively), *Ruminococcus* or *Fecalibacterium prausnitzii*.

5.2.4 Biochemical potential of the microbial community

In **Manuscript II**, the biochemical potential was evaluated based on relative abundance of functional bacterial groups, detected via Next Generation Sequencing (NGS) analysis. Similar to total microbial community (**Section 5.2.3**), also the composition of functional nitrification and denitrification-related taxa differed between Lake1, Lake2 and treated wastewater.

WWTP effluent (WW-E) presented the highest relative abundance of nitrifiers and denitrifiers, as well the highest diversity of these functional taxa. Nitrifying bacteria were mainly represented there by *Nitrosomonas* and other members of *Nitrosomonadaceae*, *Bradyrhizobiaceae* and *Rhizobiales*. The same taxa were found in wastewater recipient (point WW-R, Lake2), but not in Lake1, lacking the WWTP discharge. The actual abundance and variety of denitrifiers was more difficult to estimate because a wide range of organisms is capable of organic compounds oxidation via nitrite respiration, however they were found to belong mainly to *Bacillus, Thauera, Zoogloea, Erythrobacteraceae* and other representatives of *Alpha-, Beta-, Gamma-* or *Deltaproteobacteria*.

All this resonated well with the taxa found in treated wastewater recipient, Lake2, which to large extent mirrored the functional community of the WWTP effluent. Lake2 showed higher relative abundance of functional bacteria (<0.1%) than Lake1 (<0.01%), what indicates that treated wastewater discharge might have introduced the microbial community able to survive in the recipient and play a significant role in nutrient cycling, especially taking into account that despite the constant inflow of nutrients with WWTP effluent (eg. ≤40 mg $N\text{-}NH_4$/L), they were not accumulated in the wastewater recipient (WW-R, ≤1.2 mg $N\text{-}NH_4$/L).

In Lake1, staying under natural impact, the nitrifiers were represented by other taxa than in samples related to wastewater – they were mainly *Nitrospira* and *Candidatus Brocadia*. Denitrifiers were also less diverse than in other samples and represented mainly by genus *Flavobacterium*. Together with results described in **Section 5.2.3**, all the above suggest that wastewater discharge can increase the biodiversity of the microbial community in the receiving water body, including its functional, nutrient-cycling related part. This might be of special concern in typically oligotrophic Arctic environment, where wastewater discharge can be associated with supply of nutrients to the surface waters, but on the other hand it can also increase their biochemical potential for self-purification.

5.2.5 Sanitary status - presence of fecal indicators

Sanitary status of the treated wastewater, its recipient (Lake2) and reference Lake1 remaining under natural impact has been tested by means of cultivation on selective medium, enumeration and biochemical identification of *Enterococcus spp.* Enterococci have been chosen because of their role as fecal contamination indicators (Boehm & Sassoubre, 2014). Enterococci as indicators of environmental fecal contamination.), potentially able to survive harsh environmental conditions (Fisher and Phillips, 2009; Gaca and Lemos, 2019), as well as their increasing emergence as leading cause of nosocomial infections, including multidrug-resistant ones (Arias et al., 2010; Byappanahalli et al., 2012).

The mechanical-biological wastewater treatment applied in the Polish Polar Station's WWTP did reduce, but not completely eliminate human gut-related bacteria from the treated effluent. Thus, the WWTP effluent (WW-E) introduced a significant number of Enterococci (700 – 1,900 CFU/100 mL; CFU – colony forming units) to the recipient, what is slightly lower but falling in the range of values met in other studies of treated wastewater (Novo and Manaia, 2010; Sadowy and Luczkiewicz, 2014). Thus, the daily load of *Enterococcus* bacteria discharged with treated WWTP effluent can reach roughly between 4 – 70 mln Enterococcus CFU, depending on the number of people in the Station. Wastewater recipient (WW-R) presented lower concentrations (11 – 150 CFU/100 mL), while the lowest values were noted in Lake1 (<1 – 30 CFU/100 mL), which is lacking the wastewater discharge impact. The concentration of Enterococci in tested lakes did not exceed the limit values (≤200 CFU/100 mL; New Bathing Directive 2006/7/EC).

The composition of the enterococcal community also differed in between the samples. In Lake1, missing the treated wastewater influence, *E. fecalis* predominated, constituting for over 70% of the *Enterococcus* species (**Figure 5**). On the other hand, the wastewater-related samples showed prevalence of *E. faecium* (62.5% in WW-E, 80% in WW-R) and much smaller shares of *E. facealis* (15-25%). The reason of such discrepancy is not fully understood, but might be related to the enterococcal niche specificity, suggested by Zaheer et al., 2020, regarding e.g., broader host-range of *E. facealis* than *E. faecium*.

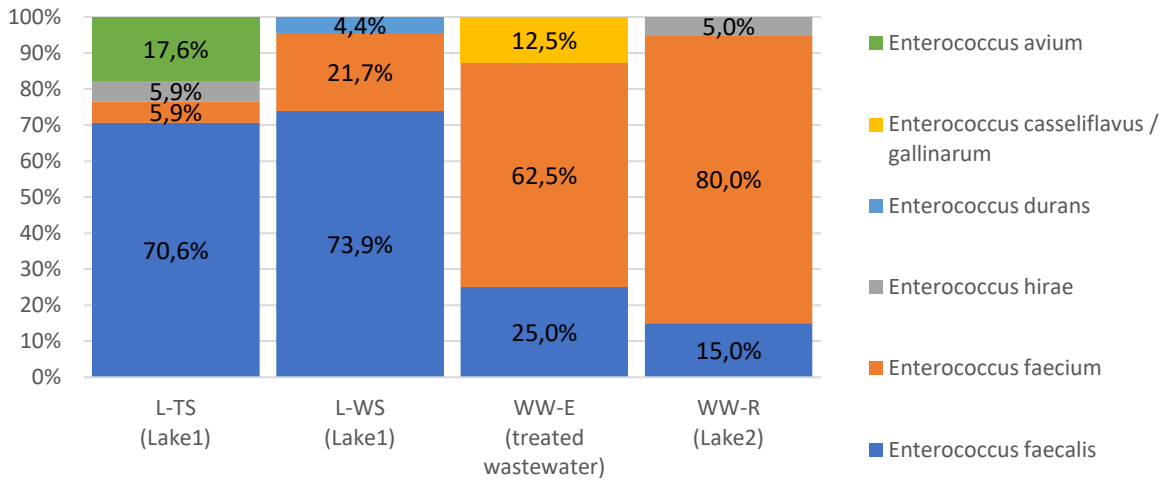

Figure 5 Identification of Enterococcus spp. isolates from tested samples

5.2.6 Antibiotic resistance of chosen fecal indicators (*Enterococcus* spp.)

For antimicrobial resistance investigation, two approaches can be applied: based on clinical breakpoints and epidemiological cut-off values (so called ECOFFs, EUCAST guedelines). The clinical approach is based on the concentrations of antibiotics used in the therapy and the probability of an infection caused by a particular bacterial strain or isolate to be treatable. The epidemiological cut-off values concept is more appropriate for the environmental studies as it is based on differentiating the wild type bacteria (likely to represent none or minimal resistance to the drug of question) from the non-wild type bacteria possessing acquired or mutational resistance mechanisms (likely to be drug-resistant). Both concepts are based on minimum inhibitory concentration (MICs), so the smallest concentration of antibiotic that is going to inhibit the growth of a microorganism. Thus, clinical breakpoints define the antibiotic concentrations at which infection is probable to be treated – this determines whether the bacterial isolate is considered susceptible or resistant. In case of epidemiological cut-off values, if the MIC for a bacterial isolate is lower than ECOFF, the isolate is likely to belong to a wild-type drug-susceptible population, while if the MIC value is above the ECOFF – the microorganism is likely to be resistant. The representatives of the same genus may exhibit different intrinsic resistance patterns, therefore, the identification to species is important and was performed for *Enterococcus* isolates in scope of **Manuscript II**, as mentioned in **Section 5.2.5**. The study focused on *E. faecium* and *E. fecalis*, as they belong to human and animal gastrointestinal microbiota (Y. Wu et al., 2019), are the most abundant enterococcal species in the wastewater (Blanch et al., 2003) and accounted for most of the identified isolates (32 and 36 isolates, respectively, out of total 76 isolates).

Among 10 tested antimicrobial agents, belonging to 9 different groups, the clinical type of resistance was detected for nitrofurantoin (used in urinary tract infections) in *E. fecalis* isolates from treated wastewater samples (**Figure 7 in Manuscript II**). For *E. faecium*, the clinical breakpoints for nitrofurantoin are not defined, and while ECOFF value is higher than for *E. fecalis* (256 mg/L versus 32 mg/L), none of the tested strains exceeded it. Therefore, most of the tested strains were found to be probably the wild type bacteria with no acquired resistance. However, some isolates with MIC above ECOFF were also noted, most frequently in treated wastewater samples. *E. faecium* was also noted to be more resistant to ampicillin than *E. fecalis*, what was in agreement with clinical studies (Jabbari Shiadeh et al., 2019). Due to limited amount of isolates, no unambiguous conclusions should be drawn, however results presented in **Manuscript II** indicate that treated wastewater contained bacteria with acquired (non-wild type) resistance to nitrofurantoin (clinical resistance in some isolates), as well as moxifloxacin and erythromycin.

5.2.7 Conclusions

Chemical, microscopic and cultivation analysis showed that the highest values of all tested parameters were found in the treated wastewater, followed by anthropogenically impacted Lake2, and the lowest for 'natural' Lake1 (**Table1 and Figure 2 in Manuscript II**). Therefore, the results of **Manuscript II** indicate that treated wastewater discharge introduces nutrient and bacterial load (related to e.g. human gut and activated sludge microbiota) to the recipient, and thus impacts both chemical and microbiological parameters of the receiving Arctic lake. Even if the nutrients from the wastewater discharge were not shown in this study to be accumulated in the Arctic lake – the combination of climate changes, e.g. warming of the Arctic, and continuous introduction of nutrients with the wastewater may result in eutrophication, microbial community shift or other issues associated with human activity.

The overall fate and function of wastewater-related bacteria in the recipient, as well as the possible implications of their release to the Arctic environment are not fully understood yet. Nevertheless, the results of **Manuscript II** showed that WWTP effluent can increase the diversity of microbial population in the receiving waterbody and introduce some taxa related to nutrient cycling. Despite very few possible sources of antibiotics at the polar station (no pharmaceutical industry, no hospital, limited population), and thus expected low concentrations of antibiotics and their residues in the wastewater treatment system(Hassoun-Kheir et al., 2020), treated wastewater contained also potentially pathogenic, human-gut related bacteria of altered resistome. The daily *Enterococcus* load released from the Polish Polar Station's WWTP ($Q \leq 3.6$ m^3/d) to the Arctic environment reached up to 7×10^6 CFU, including the strains with clinical resistance to nitrofurantoin or acquired, non-wild type of resistance to nitrofurantoin, moxifloxacin and erythromycin.

These results are particularly relevant taking into consideration the unknown consequences of such discharge for the fragile Arctic ecosystem. They also provide additional evidence of the global outreach of antibiotic resistance dissemination issue, even in areas relatively underexposed to anthropogenic impact. In these terms it would be suggested to use mitigation strategies: (1) at source - by creating a system monitoring the consumption of pharmaceuticals and usage of personal care products and substitute them if possible by environmentally friendly compounds; (2) as a final barrier – by applying the advanced end-of-pipe, locally tailored solutions, targeting precisely identified risks.

To sum up, in **Manuscript II** the applied variety of analysis revealed several aspects to take into account: temporary inability to maintain chemical parameters in the outflow at the required level and release of wastewater and human related bacterial population to the Arctic environment, with particular attention drawn to antibiotic-resistant Enterococci. Biological treatment is one of the most commonly used at polar research stations (Gröndahl et al., 2009). This type of treatment could be however supported by e.g. membrane separation, ozonation, activated carbon, UV disinfection. Above methods may serve as

mitigation strategies limiting disseminations of emerging pollutants, such as antibiotic resistant bacteria/genes (ARB/ARG) and micropollutants (including pharmaceuticals).

In the context of previous study (**Manuscript I**), growing population in the Arctic may pose the risk on the environment and this environmental impact assessment would be as important, as difficult. The data provided in **Manuscript II**, as well as further monitoring of polar areas could be used to determine the baseline conditions - the values corresponding to minimal or no human impact. Comparison with areas under larger and more diversified anthropogenic pressure could be used to assess the scale and type of hazards associated with WWTPs' effluent discharge and to feedback the wastewater treatment policies worldwide, e.g. verify the standards for wastewater discharge, especially regarding non-existing regulations on load of human-associated, potentially pathogenic fecal indicators, including the resistant ones. All these led to the studies described in **Manuscript III and IV**.

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„Badanie lekooporności, wirulencji i zróżnicowania bakterii wskaźnikowych w odpływach z oczyszczalni ścieków odbiorniku", project no. NN305461139, funded by Polish Ministry of Science and Higher Education

The above-discussed results were presented during following conferences:

1. **Kalinowska A**., Łuczkiewicz A., Jankowska K.M. „*Występowanie antybiotykooporności wśród bakterii z rodziny Enteroccoccus izolowanych ze ścieków oczyszczonych i wód powierzchniowych w rejonie polarnym (Polska Stacja Polarna, Hornsund, Spitsbergen)"*, II Interdyscyplinarna Akademicka Konferencja Ochrony Środowiska, Politechnika Gdańska, 17-20.03.2017

2. **Kalinowska A**., Łuczkiewicz A., Jankowska K.M., Fudala-Książek S. "*Arctic Lake Bacterioplankton Shaped By Anthropogenic And Environmental Factors*", International Water Association 10th Micropol & Ecohazard Conference, Wiedeń, 17-22.09.2017

3. **Kalinowska A.,** Kotlarska E., Baraniak A., Łuczkiewicz A., „*Antimicrobial resistance as a novel indicator of environmental pollution*", 10th Eastern European Young Water Professionals Conference, 7-12.05.018, Zagrzeb, Chorwacja

4. **Kalinowska A**., Jankowska K., Łuczkiewicz A., *„Treated wastewater influence on the microbial community composition of the recipient (Hornsund, Spitsbergen)",* III Ogólnopolska Konferencja Młodych Badaczy Ekosystemów Górskich i Polarnych, Karpacz, 7-9.06.2019 (1st award for the best oral presentation)

5.3 MANUSCRIPT III - INSIGHTS INTO THE MICROBIAL COMMUNITY OF TREATED WASTEWATER, ITS YEAR-ROUND VARIABILITY AND IMPACT ON THE RECEIVER, USING CULTIVATION, MICROSCOPY AND AMPLICON-BASED METHODS

The previous study shed the light on the microbial community composition of the WWTP effluents and indicated the release of nitrogen-cycling or human gut bacteria together with wastewater discharge, Similar issues have been addressed in **Manuscript III**, but in year-round context: fluctuations of community composition and its relation with chemical parameters. Additionally, reservoirs under higher anthropogenic pressure were taken into consideration.

Therefore, the results described in **Manuscript III** refer to two full-scale municipal WWTPs, working for Gdańsk and Gdynia agglomerations, located in Northern Poland: WWTP Gdańsk-Wschód (WWTP-W) and WWTP Gdynia-Dębogórze (WWTP-D), respectively. Both WWTPs combine conventional mechanical step with advanced biological nutrient removal (occasionally supported by chemical precipitation for the phosphorus removal; **Table 3**). For both of WWTPs, approximately 10% of the inflow accounts for industrial wastewater; mostly from the food or chemical industry (including pharmaceuticals and cosmetics) and shipyards.

For **Manuscript III**, treated wastewater (TW) was collected for 12 consecutive months, together with occasional sampling of wastewater discharge points - marine outfalls (MO: MO-D for WWTP-D and MOW for WWTP-W effluent outflow) and reference points: at the open sea, Gdańsk Deep station (GD) far from anthropogenic impact and at the estuary of Vistula River (VIS), which receives treated wastewater along its course. In scope of **Manuscript III**, the chemical and microbiological characteristics of treated wastewater (TW), its recipient at points of discharge (MO) and reference points (VIS, GD) were analyzed (**Figure 6**). The microbial community composition, its quantity and biochemical potential were investigated in the samples, together with the sanitary status. Special attention was given to the year-round variability of chosen parameters.

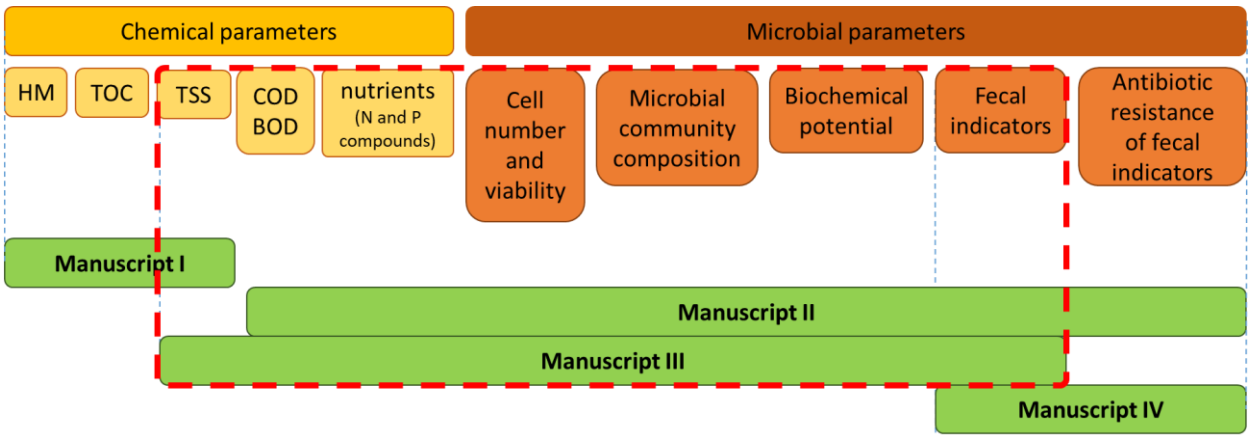

Figure 6 Schematic overview of the parameters tested in Manuscript III

5.3.1 Chemical parameters

The chemical characterization of the treated wastewater done in scope of **Manuscript III** included the standard parameters (COD, BOD, TSS, TN, TP) required by Polish legislation in monitoring the quality of WWTP effluents (Dz.U. 2019 poz. 1311) which are summarized in **Table 8**. Values were typical for the municipal WWTPs (Krzeminski et al., 2012; Pasztor et al., 2009; Szklarek et al., 2021). Both WWTPs showed high and relatively stable removal efficiency (on average 90-99%, depending on the parameter, **Table 8).** Even though the principal component analysis (PCA) revealed that influent parameters did not significantly differ between the WWTPs (Supplementary Figure S1, Manuscript III), the effluent samples were clearly separated with respect to WWTP of origin. The parameters that most differentiated the treated wastewater from the two treatment plants were COD and the concentrations of nitrogen and phosphorus compounds (**Figure 3 in Manuscript III, Supplementary Figure S2 in Manuscript III**). The differences between WWTPs effluent may result from discrepancies in treatment processes management: e.g. seasonal adjustments done by operators, different size of WWTPs or slight variations in applied nutrient removal systems

Quality of the final effluent showed clear relation to ambient temperature (**Figure 3 Manuscript III**). Despite seasonal fluctuations in effluent parameters, the values did not exceed the norms required by Polish legislation for treatment plants of this scale (Dz. U. 2019, poz. 1311, PE ≥ 100 000; **Table 8**). However, deterioration of chemical parameters of the treated wastewater indicates disturbance in activated sludge functioning. This results in small flocs formation and thus worsening of bacterial biomass sedimentation and washout of free-floating cells with treated effluent, what has been observed in microscopic analysis (**See Section 5.3.2**).

Treated wastewater was well diluted in the marine recipient, thus the concentrations of TSS, phosphorus and nitrogen compounds in the wastewater receiver were significantly lower than in the WWTP effluent itself (**Table 8**). Concentrations of COD and BOD in environmental samples (MO, VIS) showed some seasonal differences and were higher in summer than in winter, reaching values similar to treated wastewater. Interestingly, samples from the Vistula estuary showed similar or slightly higher values of the tested parameters than the marine outfalls of the wastewater. This can be explained by the eutrophication of Vistula River itself (Maksymowska et al., 2000), either due to wastewater discharge or intensively cultivated basin area, resulting in increased concentrations of nutrients.

Table 8 Chemical characteristics of treated wastewater and environmental samples tested in scope of Manuscript III (Kalinowska et al. 2022): WWTP-W stands for WWTP Gdańsk-Wschód and WWTP-D for WWTP Gdynia-Dębogórze. Data given as mean values ± standard deviation. Table adapted from Manuscript III. Physicochemical data for Gdansk Deep (GD) are unavailable.

Parameter		pH	TSS	COD	BOD	TN	N-NH_4	N_{org}	N-NO_3	N- NO_2	TP	P-PO_4
Unit		[–]	[mg/dm^3]	[mg O_2/dm^3]		[mg N/dm^3]					[mg P/dm^3]	
Removal efficiency [%]	**WWTP-W**	–	98.9±0.7	96.7±0.7	99.3±0.3	91.1±1.6	99.1±0.3	–	–	–	96.9±1.1	99.0±1.3
	WWTP-D	–	98.7±0.5	97.5±1.7	99.2±0.2	90.7±1.4	98.9±0.3	–	–	–	93.6±3.5	91.1±6.6
Minimum removal efficiency* [%]		–	90	75	90	70–80	–	–	–	–	80	–
Effluent quality	**WWTP-W**	7.9±0.15	5.2±2.67	33±4.1	2.9±1.2	7.8±1.2	0.6±0.21	1.7±0.64	5.6±0.9	0.07±0.06	0.3±0.12	0.08±0.06
	WWTP-D	7.8±0.15	5.3±0.83	26±5.4	3.4±0.8	8.3±1.2	0.7±0.22	1.5±0.37	6.1±1.07	0.06±0.04	0.7±0.36	0.48±0.35
Maximum permissible values for treated wastewater* [mg/L]		–	35	125	15	10	–	–	–	–	1	–
Marine recipient at point of wastewater discharge	**Summer**	–	2.6±0.9	30±13	2.5 ±0.96	0.5±0.16	0.03±0.01	–	–	–	0.07±0.02	0.02±0.01
	Winter	–	3.2±1.9	0.5±0.37	<LOD	0.4±0.07	0.03±0.01	–	–	–	0.12±0.03	0.08±0.01
Vistula River estuary	**Summer**	–	2.6	32.8	3.6	0.09	0.04	–	–	–	<0.5	0.11
	Winter	–	3.9	0.40	<LOD	0.85	<0.015	–	–	–	<0.5	<0.05

*requirements for municipal wastewater discharged into waters or into the ground from WWTPs >100 000 people equivalent, according to Polish standards (Dz.U. 2019 poz. 1311)

5.3.2 Quantification of the prokaryotic community and its viability

On average, both WWTPs showed similar values of prokaryotic abundance and biomass in the effluent (2.21 and 2.22 mln cells/mL and 68.27 versus 69.22 µg C/L for WWTP-W and WWTP-D, respectively) with seasonal variability, indicating lower quality of the discharged effluent in winter months (higher bacterial abundance, smaller cells structured into small flocs, biomass washout, presence of foaming and bulking bacteria and human gut related, potentially pathogenic species, for details see also **Section 5.3.3. and 5.3.5**, **Figure 6 in Manuscript III** or **Supplementary Figure 3 in Manuscript III**).

All the parameters obtained via microscopic analysis (total prokaryotic cell number (TCN), prokaryotic biomass (PB), average cell volume (ACV) and live cells percentage (LD) were on average higher for treated wastewater than in environmental samples (**Figure 7**). In environment, the mean values and dispersion of results was similar for marine outfalls (MO) and Vistula estuary (VIS), while consequently lower the reference point at the open sea - Gdańsk Deep (GD), which is devoid of the direct anthropogenic impact associated with wastewater discharge. WWTP effluent contained on average the largest cells - around 0.15 µm^3, while in environment the average cell volume (ACV) was two times lower (0.06-0.08 µm^3). Also in terms of cell viability, treated wastewater showed higher share of live cells (LD between 10-23%) than in environmental samples (8-13% at MO, 9-11% at VIS). This is possible due to the activated sludge origin of the cells in WWTP effluent, as the active population in the reactor can reach even 80% (Kocwa-Haluch and Woźniakiewicz, 2011).

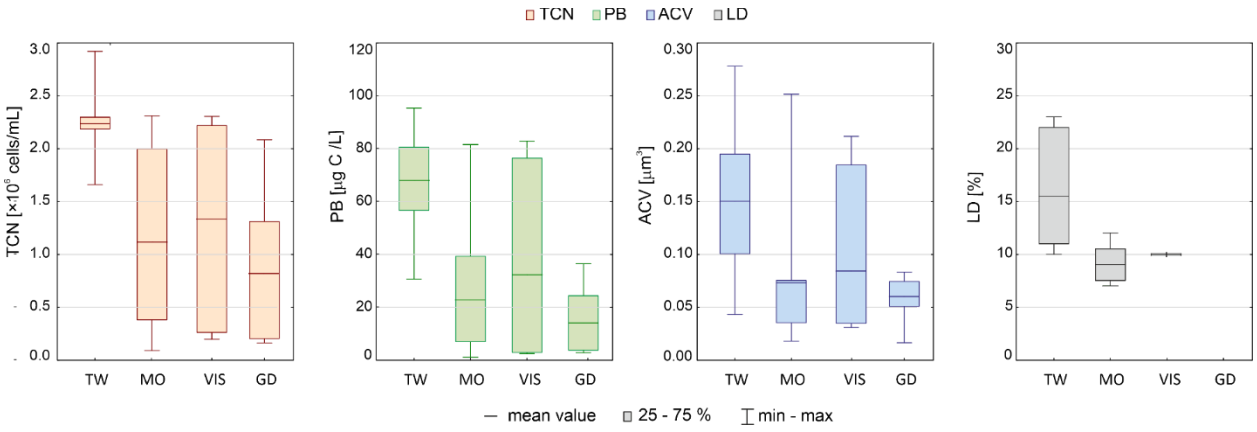

Figure 7 Results of the microscopic analysis. Figure adapted from Manuscript III.

5.3.3 Microbial community composition

The microbial community of the treated wastewater on phylum level showed great similarity between WWTPs (contained 22 same core phyla), although large variations were observed in the percentage abundance of individual taxa throughout the year (**see Fig 5a,b in Manuscript III**). Taxonomic composition of the effluent to some extent resembled treated wastewater described in other studies, e.g. from Ireland (Do et al., 2019), Belgium (García-Armisen et al., 2014), Estonia (Tiirik et al., 2021), Hong Kong (Cai et al., 2014) or North America (Xue et al., 2019) and reflected the typical composition of activated sludge (L. Wu et al., 2019). This suggests a washout of AS related bacterial biomass out of the bioreactor, especially in winter months, when chemical and microbial quality of the effluent usually decreases (**see sections 5.3.1 and 5.3.2**). This was also confirmed by metagenomics approach and canonical correspondence analysis (CCA, **Figure 6 in Manuscript III**). Treated wastewater samples from months of lower ambient temperature, corresponding to worse phosphorus removal and higher nitrogen concentration, were also characterized by elevated levels of bacteria potentially responsible for foaming and bulking (*Candidatus Microthrix*), related to human gut (*Carnobacteriaceae*, *Lachnospiraceae* or *Gordonia*) or potential pathogens (*Campylobacteraceae* and *Legionellaceae*). These results are consistent with microscopic observations: different flocs appearance and increased cell abundance and biomass found in the treated wastewater in winter samples (**Section 5.3.2**). Additionally, also the microbial diversity of the effluent was lower in winter, what is in agreement with findings of Wang (et al., 2012) in bioreactors.

Despite the seasonal fluctuations in microbial diversity, it was on average the highest in treated wastewater samples, followed by sampling stations at wastewater discharge (MO) and Vistula estuary (VIS), and the lowest for open sea (GD), **for details see Figure 5d in Manuscript III**). This is in line with other studies showing that wastewater discharge can enrich the bacterial community of the recipient (García-Armisen et al., 2014; Price et al., 2018), and reflects mixing of fresh and brackish waters communities at Vistula mouth. The taxonomy composition of environmental samples was different than treated wastewater (**for details see Figure 5a in Manuscript III**), regardless of the presence of wastewater impact. The most characteristic phyla of environmental samples were *Cyanobacteria*, *Planctomycetes* and *Verrucomicrobia* constituting for even 54% of their microbial community, while in treated wastewater these taxa did not exceed 3.5%. Even if the environmental samples contained some of the phyla dominating also in the WWTP effluent (*Actinobacteria*, *Bacteroidetes* or *Proteobacteria*), they could also be associated as taxa accompanying cyanobacteria blooms (Berg et al., 2009).

5.3.4 Biochemical potential of the microbial community

In this study, the biochemical potential was investigated in two ways – similarly as in **Manuscript II**, via microbial community composition analysis (obtained with next generation sequencing, NGS), and also more specifically, with respect to abundance of selected nitrogen cycle genes (*amoA*, *nxrA*, *nirS*, *nirK* and *nosZ*), using quantitative real-time polymerase chain reaction (PCR).

Analysis of sequencing data revealed that nitrification and denitrification community was on average more abundant and more diversified in treated wastewater than in environmental samples. It was also represented by different taxa: in WWTP effluent the nitrifiers were represented mainly by *Nitrosomonas* (ammonia-oxidizing bacteria, AOB) and *Nitrospira* (nitrite oxidizing bacteria, NOB, with potential comammox ability), while *Nitrospumilus* (ammonia-oxidizing archaea, AOA) and *Nitrospina* (NOB) prevailed in the coastal and marine samples (with occasional presence of *Nitrospira* at MO and VIS sampling stations). Nitrifiers were also more abundant at points of wastewater discharge (MO, <0.44%) than at Vistula estuary (VIS, <0.04%) or open sea (GD, <0.02%). In case of potential denitrifiers, a wide range of *Protobaceteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* was taken into consideration: in treated wastewater the most abundant were *Acinteobacter*, *Dokdonella*, *Dechloromonas*, *Hyphomicrobium* and others (on average 35 taxa, 13% of total community), while in environmental samples *Flavobacterium* and *Rhodobacter* clearly dominated, with minor shares of other taxa (av. 24 taxa, 6.4% of total community).

In quantitative approach, selected genes have been used as molecular markers of nitrification (*amoA*, *nxrA*) and denitrification (*nirS*, *nirK* and *nosZ*). Their functions in the nitrogen cycle are schematically shown in **Figure 8**. For all the tested genes, the average abundance of gene copies per 1 mL sample (gc/1 mL) was always the highest in treated wastewater (TW) and the lowest for GD (**for details see Fig. 7 in Manuscript III**). Like in the other analyses (**Sections 5.3.1 – 5.3.3., 5.3.5.**), the MO and VIS sampling points showed similar ranges and 'middle' values of gene abundance.

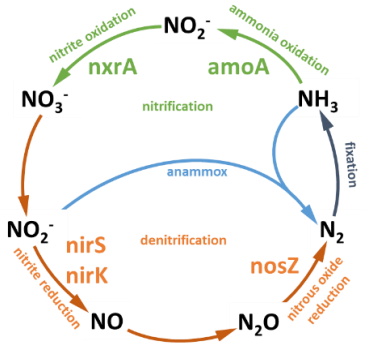

Figure 8 Simplified schematic diagram of the nitrogen cycle and functions of selected genes quantified in scope of Manuscript III

For nitrification, representative genes *amoA* and *nxrA* were chosen as process markers. Since no anammox bacteria were found in any of the samples, and ammonia-oxidizing archaea (AOA) were found ocasionally only in MO and GD samples, the *amoA*-based community should be linked mainly to ammonia-oxidizing bacteria (AOB) – *Nitrosomonas* and other representatives to *Nitrosomonadaceae* family (detected via NGS). Abundance of amoA ranged between 10^4-10^5 gene copies/1 mL sample at TW, 10^3-10^5 gc/1 mL sample at MO, 10^3-10^4 gc/1 mL sample at VIS and 10^3 gc/1 mL sample at GD (**for details please see Fig. 7 in Manuscript III**). Gene nxrA, encoding nitrite oxidation, was one magnitude lower and assigned to presence of NOB form family *Nitrospiraceae* (prevalent genus *Nitrospira* in treated wastewater and *Nitrospina* in environmental samples).

Representative denitrification markers: *nirS* and *nirK* genes presented abundance range similar to each other in each sample type (10^5-10^6 gc/1 mL sample for TW, 10^3-10^4 gc/1 mL sample for MO, 10^4-10^5 gc/1 mL sample for VIS and 10^4 gc/1 mL sample for GD). Genes *nirS* and *nirK* were also the most abundant from all the tested genes. They are mutually exclusive in terms of presence in the same organism, and it is consistent with wide range of potential denitrifiers. Gene *nosZ*, encoding the last step of denitrification – nitrous oxide reduction, was present in all the samples in range 10^4 - 10^5 gc/1 mL, except for GD samples (10^2-10^3 gc/1 mL).

The results were compared with literature data (for details see **Fig. 8 in Manuscript III**) and showed lower abundance than in the activated sludge, investigated in other studies (e.g. Che et al., 2017; Cole et al., 2004; Geets et al., 2007; Wang et al., 2012). It is worth noting that in environmental studies, the gene abundance was tested most frequently in soil or sediments, rather than in waters of the recipient. To the best of author's knowledge, **Manuscript III** is one of the first studies concerning nitrogen cycling genes abundance in WWTP effluent and its recipient.

5.3.5 Sanitary status - presence of intestinal bacteria

Sanitary status of the treated wastewater, its recipient at the points of wastewater outfalls (MO-W and MO-D) and at the reference points (VIS, GD) has been investigated by cultivation on appropriate selective media and quantification of intestinal bacteria (*Enterobacteriaceae*), fecal coliforms and their major representative, *Escherichia coli*. These bacteria have been chosen due to their importance in context of human health and role as fecal indicators in the environment. For example Enterobacteria group contain opportunistic nosocomial pathogens (such as *Escherichia*, *Shigella, Klebsiella* or *Acinetobacter*) of growing significance due to the global development of their multidrug resistance (Urase et al., 2020).

Likewise, the microscopic analysis (**Section 5.3.2.**), the cultivation results revealed a high similarity between the WWTPs in terms of sanitary status of their effluents. However, WWTP-W presented on average lower values than WWTP-D effluent with respect to all tested parameters: abundance of *Enterobacteriaceae* (3.6×10^5 for WWTP-D and 3.2×10^5 CFU/100 mL for WWTP-W), fecal coliforms (10.4×10^4 and 9.2×10^4 CFU/100 mL, respectively) and *E. coli* (2.9×10^4 and 2.6×10^4 CFU/100 mL, respectively, **Fig. 9**). These values fell in the ranges reported for treated wastewater by other studies (Korzeniewska et al., 2013; Korzeniewska and Harnisz, 2012; Łuczkiewicz et al., 2010; Marano et al., 2020).

In environmental samples, tested bacterial groups were on average two to three magnitudes lower than in treated wastewater (**Figure 9**). This indicates that despite wastewater treatment processes usually represent high removal efficiency of the fecal bacteria (>90%, Lucena et al., 2004; Marano et al., 2020), considerably high amounts of human gut related microorganisms are still discharged to the environment with WWTP effluent, due to their high population in raw wastewater. Nevertheless, the permissible standards for *E. coli* in the surface waters were not exceeded (<500 CFU/100 mL; new Bathing Water Directive, 2006/7/EC). Marine outfalls presented higher values of bacterial indicators than the reference point at the open sea - Gdansk Deep (**Figure 9**), but similar or lower to Vistula River estuary. This supports selecting Gdansk Deep as a reference sampling station, being isolated from the freshwater or anthropogenic (e.g. wastewater-related) impact, but also indicates the importance of the Vistula River quality and general riverine influence on the coastal waters.

Enterococcus bacteria (fecal indicators tested in **Manuscript II**) were not cultivated in this study, but their presence was confirmed by NGS analysis – they did not exceed 0.03% of the total community in treated wastewater and were occasionally found in the waters of the recipient, but only at marine outfalls.

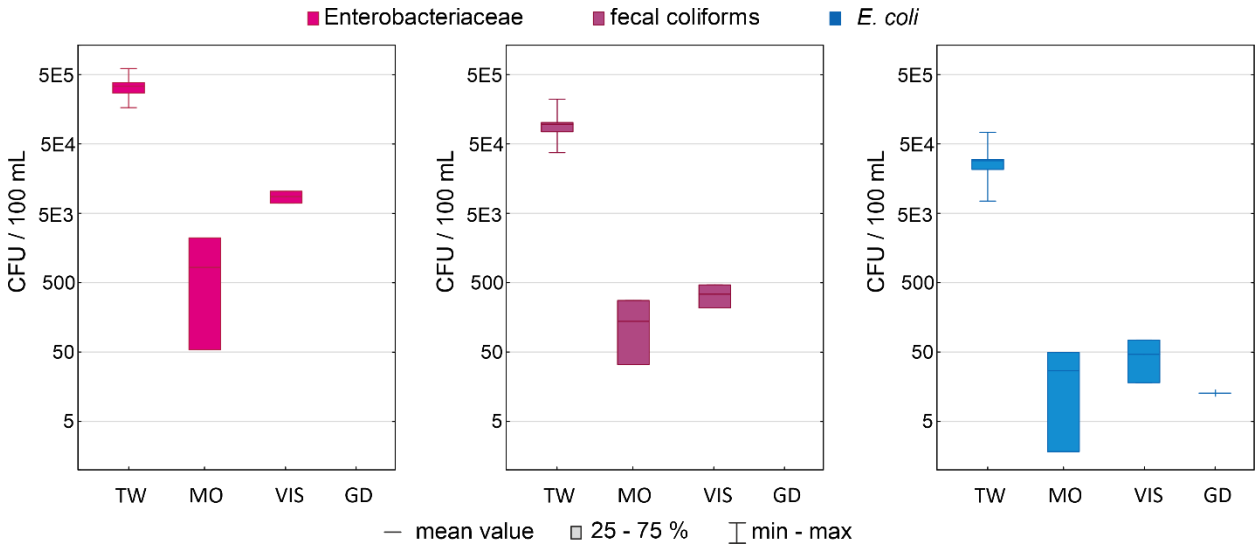

Figure 9 Results of the cultivation analysis. Figure adapted from Manuscript III.

5.3.6 Conclusions

The results of **Manuscript III** showed that the microbial community of the treated wastewater to some extend mirrored typical activated sludge composition, but its composition also underwent changes throughout the year. WWTP effluent from winter contained elevated number of small prokaryotic cells clumped into flocks, what corresponds to seasonal disturbances in activated sludge functioning and thus shows as decrease of treatment efficiency. At the same time, the deterioration of effluent quality manifested itself as an increased presence of bacterial groups associated with bulking and foaming of activated sludge or belonging to human gut (e.g. *Carnobacteriaceae*, *Lachnospiraceae* or *Gordonia*).

Thus, despite high treatment efficiency (90-99%, depending on the parameter) and meeting discharge quality requirements, it can be stated that conventional WWTPs still release the chemical and microbial constituents into the receiving waters. Sampling points related to wastewater discharge were characterized by on average double values of total cell number (TCN) and prokaryotic biomass (PB), as well as elevated average cell volume (ACV) and live cells percentage (LD), than the reference points not subjected to such impact. Similar to **Manuscript II**, it was noted that treated wastewater can increase the microbial diversity and biochemical potential of the receiver by discharge of community related to activated sludge, potentially involved into nutrient cycling.

According to author's best knowledge, **Manuscript III** is one of the few available studies (Mansfeldt et al., 2020; Pandit et al., 2021; Price et al., 2018) investigating the microbial composition of the treated

wastewater and its impact on the receiving environment. It is also one of the first published studies quantifying the nitrogen-cycle genes in WWTP effluent and in its marine recipient. Further research would reveal the dynamics of microbial communities of WWTP effluents and their adaptation after being released to the environment - in order to predict what is actually discharged with treated wastewater and recognize, what function it may play in the recipient.

Compared with **Manuscript II**, the case study described in **Manuscript III** was far more complex: from much larger population, through the variety of other anthropogenic activities in the study area or influencing it (e.g. transport, agriculture, surface runoff), to the size and dynamics of the recipient (stratification and mixing of coastal marine waters, inflow of riverine waters). In this case, the impact of a specific factor (e.g. wastewater discharge) is difficult to be distinguished. Thus, the combination of various complementary analytical methods has been used in this study to investigate various aspects of the treated wastewater discharge to the environment and to get the holistic overview on this subject. This synergistic approach has been further enhanced by application of multivariate statistical techniques, expected to identify the key parameters useful in monitoring of the WWTP effluent quality and its impact on the recipient. The surveillance itself, should be, however, less complex, universal, easy to perform and possibly low-cost. Thus, the simplistic and informative monitoring method, based on the cultivation, has been developed and tested in **Manuscript IV**.

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The above-discussed results were presented during following conferences:

1. **Kalinowska A.**, Pierpaoli M., Jankowska K., Fudala-Książek S., Remiszewska-Skwarek A., Łuczkiewicz A., *Structure And The Biochemical Potential Of The Bacterial Community In Wastewater Treatment Plant Effluent And In Receiving Waters: Northern Poland Case Study*, 12th MICROPOL & ECOHAZARD CONFERENCE 2022, 6-10.06.2022, Santiago de Compostela, Spain

2. **Kalinowska** A., Jankowska K., Fudala-Książek S., Pierpaoli M., Łuczkiewicz A., *Wpływ zrzutu ścieków na strukturę taksonomiczną, antybiotykooporność oraz potencjał biochemiczny populacji bakteryjnych w wodach odbiornika*, VI Interdyscyplinarna Akademicka Konferencja Ochrony Środowiska, 22-24.09.2021, Gdańsk, Poland

5.4 MANUSCRIPT IV - A GLOBAL MULTINATIONAL SURVEY OF CEFOTAXIME-RESISTANT COLIFORMS IN URBAN WASTEWATER TREATMENT PLANTS

Due to global importance of bacterial antibiotic resistance occurrence and spreading associated with wastewater treatment processes, **Manuscript IV** presents a transnational study focusing on presence of antibiotic-resistant fecal coliforms in the treated effluent. Thus, it complements the picture of wastewater discharge from large-scale WWTPs shown in **Manuscript III**. **Manuscript IV** combines the data from 57 WWTPs located in 22 countries on 5 continents. Most of them (47 WWTPs) were located in Europe, 2 in North America, 6 in Asia and 1 in Africa and Australia, representing various climatic zones. The study also includes data from WWTPs described in **Manuscript III**: Gdańsk-Wschód (WWTP-W) and Gdynia-Dębogórze (WWTP-D).

Results of **Manuscript IV** show the abundance of total and fecal coliforms in the wastewater (**Figure 10**) and their resistance to cefotaxime (CTX), a beta-lactam antibiotic chosen due to its clinical relevance. Cefotaxime-resistant coliforms are associated with ESBLs, extended-spectrum beta-lactamases which are enzymes capable of disrupting the antibiotic structure and thus providing the antibiotic resistance to the host bacteria. ESBLs are increasingly prevalent in environment and often linked to the occurrence of multidrug resistance (Bradford, 2001; Pitout and Laupland, 2008). Coliforms were tested also due to their wide and universal applicability as fecal contamination indicators, simplicity of cultivation procedure and thus existing standard monitoring routine, frequently applied to monitor the microbial quality of surface water reservoirs or wastewater treatment efficiency (Tallon et al., 2005). Despite the universality and simplicity of the existing monitoring of fecal indicators, it does not investigate the antibiotic resistance levels – a new global threat that requires routine assessment and is clinically relevant for coliforms due to prevalence of horizontal gene transfer of mobile genetic elements, possibly facilitating e.g. antibiotic resistance dissemination among them (Popa and Dagan, 2011).

Therefore, due to lack of universal, standard monitoring procedure of antibiotic resistance among bacteria discharged with WWTP effluents, **Manuscript IV** aimed to test the applicability of the low cost, easy to implement antibiotic resistance surveillance, based on coliforms cultivation on mFC Agar medium with or without antibiotic (cefotaxime) addition. Simultaneously, the quantification of total and cefotaxime-resistant coliforms in raw and treated wastewater enabled to estimate the reduction potential of wastewater treatment processes.

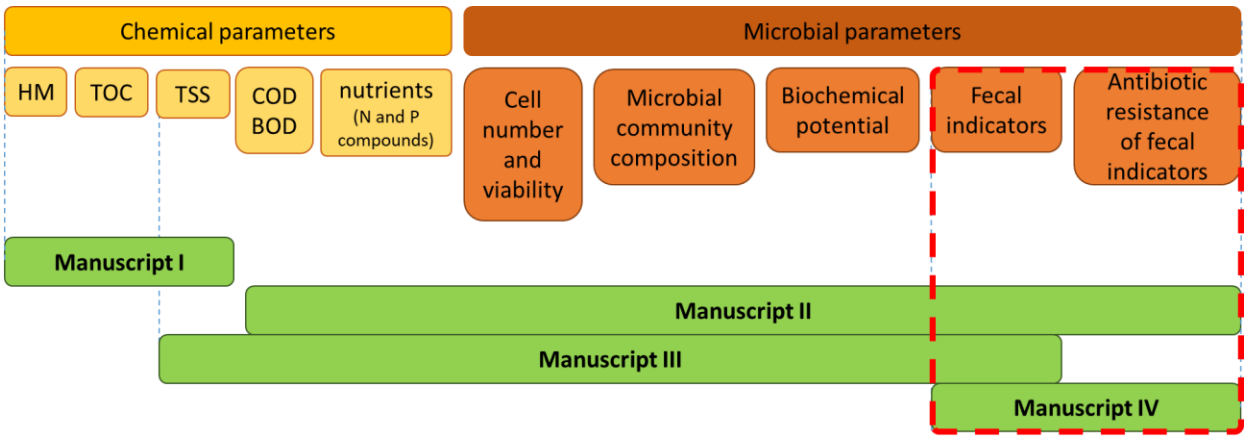

Figure 10 Schematic overview of the parameters tested in Manuscript IV.

In order to detect also the cefotaxime-resistant (CTX-R) coliforms. the existing procedure for fecal coliforms cultivation (at 44°C) was adapted in scope of **Manuscript IV**. The alternative incubation temperature of 37°C was applied to avoid the problems shown to occur in higher temperatures, e.g. curing of plasmids harboring antibiotic resistance genes (Trevors, 1986) or thermal instability of enzymes hydrolyzing beta-lactam antibiotics (He et al., 2016). To validate the proposed alternative procedure, the parallel series of tests were done on medium with and without cefotaxime at different incubation temperatures (37°C and 44°C, respectively). Cultivation method was supplemented and supported by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) or 16S rRNA gene sequence analysis (as specified in **Table 4, Section Methodology**; detailed protocols are described in **Supplementary Materials of Manuscript IV**).

From each validation test, 30 representative colony forming units (CFU) were selected, isolated and taxonomically characterized. The validation was done at the samples from 8 different geographical locations (Australia, Croatia, Germany, Israel, **Poland** [samples from WWTP-D, introduced in **Manuscript III**], Portugal, UK and USA).

5.4.1 Sanitary status - presence of total and fecal coliforms

During the validation process, over 31% of chosen fecal coliforms isolates incubated in 37°C and >71% incubated in 44°C were identified as *E. coli*. Although the percentage of *E. coli* among the tested isolates was lower for 37°C than for 44°C, the values were within the same order of magnitude, when expressed as CFU/mL. Isolates other than *E. coli*, obtained from plates incubated in 44°C still belonged to *Enterobacteriaceae* family and included potential opportunistic human pathogens such as *Citrobacter, Enterobacter* and *Klebsiella* spp. On plates incubated at 37°C the non-*Enterobacteriaceae* bacteria (mainly *Aeromonas* spp.) constituted for 23% of all isolates. Apart from the validation experiments, the taxonomy of the isolates was not additionally verified or confirmed, therefore, unless specified differently, the calculated isolates are referred to as fecal coliforms.

The results presented in **Manuscript IV** showed that despite high efficiency of wastewater treatment systems in terms of fecal coliforms removal (>99% removal was noted for 64% of the secondary treatment and 91% of the tertiary treatment effluents, for details see **Supplementary Materials of Manuscript IV**), the WWTPs still release significant loads of coliforms with their final effluents. The concentration of total coliforms ranged from <1 CFU/mL up to 1.1×10^6 CFU/mL in secondary treatment effluent, and up to 5.2×10^5 CFU/mL in tertiary treatment effluent. Fecal coliforms constituted for a highly variable fraction of total coliforms, reaching 5.6×10^5 CFU/mL in secondary treatment effluent (av. 1.7×10^4 CFU/mL) and 1.8×10^5 CFU/mL in tertiary treatment effluent (av. 2.5×10^3 CFU/mL). Compared to other WWTPs, effluents from WWTP-W and WWTP-D presented average concentrations of total and fecal coliforms: 1.2 – 17×10^3 CFU/mL for total coliforms, and 0.8 – 6.3×10^3 CFU/mL for fecal coliforms, respectively. All the aforementioned results are presented in **Supplementary Materials of Manuscript IV**. Current regulations for acceptable faecal coliform counts in WWTP effluents are usually defined only in context of water reuse and are highly variable depending on effluent application (irrigation, flushing the toilet, recreation or irrigation: either food or non-food crops), region (2.2 – 10,000 CFU/100 mL, EPA report 2012) or legislator (1000 CFU/100mL by WHO, 200 CFU/100 mL by EPA (US EPA 1976)). Despite the fact that most of the tested effluent samples contained low levels of resistant coliforms, it is alarming that still 37% of the treated wastewater samples exceeded the WHO guidelines even in terms of only CTX-R coliforms abundance (the resistance was not even considered by the WHO standard).

In order to estimate the reduction efficiency of wastewater treatment processes, bacteria were quantified also in the raw wastewater. Collected data revealed that coliforms were more abundant in raw wastewater from areas with higher monthly ambient temperature (two data subsets were identified: below and over 15°C). Nevertheless, the observed correlation may also result from a variety of interrelated socioeconomic conditions, such as existing wastewater network, hygiene or population density. Higher

coliforms concentrations in the influent were suspected to favour higher bacterial load at the WWTP outflow, however no significant overall differences in coliform removal were observed in respect to the ambient temperatures. This is also in agreement with results of **Manuscript III**, where no relationships were found between season and fecal coliforms concentration at the WWTP outflow or between influent and effluent quality. The limited role of the influent quality was also shown and discussed by (Blanch et al., 2003; Novo and Manaia, 2010; Vilanova et al., 2002), where the treated effluent composition was related to the characteristics of the treatment plant, e.g. its size or treatment setup.

The results presented in **Manuscript IV** indicated that the average global reduction of coliforms was 2.3±1.2 log removal by secondary treatment methods, however membrane bioreactors (MBR) were significantly more efficient than conventional activated sludge (CAS): mean 5.8 ± 0.6 and 2.1 ± 0.8 log removal, respectively. MBR efficiency may result most likely from the small pore size, especially if the ultrafiltration is applied, however MBR effluent data were far less common (n=8) than from CAS systems (n=140). Disinfection (namely: ozonation, chlorination, UV radiation, or combination of the two latest) was applied in only 7 out of 57 tested WWTPs and increased the coliforms removal to 4.4±2 log units (worst removal efficiency of tertiary treatment was 92%, while of secondary treatment – 21%). Although both the secondary and tertiary treatment stages were also able to reach similarly high removal efficiency (>99.9%), the incidence of such great performance was different for different treatment methods and occurred in 19% results (36 out of 188) of secondary treatment and for 75% results (49 out of 65) of tertiary treatment. For WWTP-D and WWTP-W the removal efficiencies ranged between 93.2 – 96.6% for total coliforms removal, and 96.6 – 99.7% for fecal coliforms removal.

5.4.2 Antibiotic resistance of total and fecal coliforms

In the validation part, the incubation of plates with cefotaxime in 37°C showed significantly higher recovery of cefotaxime-resistant (CTX-R) *E. coli* than in the procedure conducted at 44°C. This supports the hypothesis of acquired resistance being more stable at lower incubation temperature (Trevors, 1986), and certifies the incubation at 37°C for targeting the resistant *E. coli* (fecal coliforms) from wastewater, despite generating slightly elevated number of false positive isolates (presumptive *Enterobacteriaceae* being non coliforms or aeromonads). Incubation in 44°C might under-select the CTX-R coliforms or make them loose the resistance determinants (e.g. plasmids), leading to bias in their evaluated abundance in the wastewater sample. Therefore, in this method, designed to target the antibiotic resistance of fecal coliforms, the recommended temperature for cefotaxime-supplemented plates incubation is 37°C.

The concentration of cefotaxime-resistant (CTX-R) **total** coliforms ranged from <1 CFU/mL up to 6.6×10^4 CFU/ mL in secondary treatment effluent (av. 1,500 CFU/mL), and up to 1.2×10^4 CFU/mL in tertiary treatment effluent (av. 500 CFU/mL). CTX-R **fecal** coliforms ranged from <1 CFU/mL to 3.0×10^4 CFU/mL (av. 358 CFU/mL) in treated wastewater after secondary treatment. In WWTP-W and WWTP-D the disinfection was not applied, and the concentrations of CTX-R coliforms in the final effluent always oscillated around or below global mean values for secondary treatment: for total resistant coliforms they ranged between approx. 15 – 1,300 CFU/mL and 50 – 500 CFU/mL, respectively, and between 5 – 500 CFU/mL and 20 – 200 CFU/mL for resistant fecal coliforms, respectively. Tertiary treatment resulted in reduction of CTX-R coliform load of the final effluent, with concentrations reaching maximum 1.0×10^3 CFU/mL (av. 24 CFU/mL).

Despite the reduction rates being usually over 99% (150 out of 220 recordings), this is considerable load of cefotaxime resistant bacteria being released to the environment. The most frequently applied method among all the studied WWTPs, based on conventional activated sludge (CAS) treatment, removed approx. 2 log units of CTX-R coliforms from the wastewater. Disinfection increased the removal to 3.5±1.4 log units and 35% of the analysed disinfected samples showed abundance of CTX-R coliforms in the final effluent below limit of detection (0.3 CFU/mL), but still a large result dispersion was noted for disinfected effluents in terms of both total and CTX-R coliforms.

Due to the largest number of observations, the secondary treatment's removal efficiency of coliforms and CTX-R coliforms was also investigated as a function of regional ambient temperatures (on temperature subsets <5°C and >15°C, for details see **Supplementary Figure 7 and 8 in Manuscript IV)** to evaluate the possible relation between removal efficiency and temperature. Overall, no significant differences were observed between the two subsets, however for lower temperature subset (<5°C), a higher number of WWTPs with lower removal efficiencies (<95%) of CTX-R coliforms were noted. Lowest efficiencies were

observed in some, but not all WWTPs from Poland, Germany, Estonia, Austria and UK, suggesting that instead of being a national or geographical trend, these might be a sporadic failures of the system, where the temperature influence has not been clearly confirmed or rejected.

Similar to the results presented in **Section 5.4.1.**, CTX-R coliforms were also quantified in raw wastewater. Yet again, resistant bacteria were most abundant in raw wastewater from areas with higher ambient temperatures, however no statistical significance with temperature was found. Apart from the fact that correlation does not necessarily mean causation, this relation may be also stemming from a myriad of other factors, more related to socioeconomic characteristic of the area, that were not targeted in this study, e.g. fragile sanitary infrastructure, standards of hygiene or unregulated antibiotic production and consumption. Average defined daily doses (DDD) of beta-lactam antibiotics were taken into account in this study, but they did not provide any meaningful explanation for coliform or CTX-R coliform number in raw sewage. Previous studies (Caucci et al., 2016; Collignon et al., 2018) indicated that national antibiotic consumption data are usually of low resolution and thus may not reflect the local conditions for given WWTP. Also contribution of hospital effluents (measured as ratio of bed equivalent to population served) did not correlate with CTX-R coliforms. This suggests that antibiotic resistance may be governed by complex ecological interactions that cannot be explained based only on antibiotic consumption or even presence of antibiotic residues in wastewater.

5.4.3 Conclusions

In scope of **Manuscript IV**, wastewater from 57 WWTPs worldwide was investigated in terms of coliforms and cefotaxime-resistant (CTX-R) coliforms abundance, as well as their removal efficiencies. The results show that despite high bacteria removal rates (>99%) shown by many WWTPs, their effluents still supply the continuous load of coliforms and CTX-R coliforms to the receiving waters, what may play a role in further dissemination of antibiotic resistance in the environment.

On average, the secondary (CAS and MBR) treatment reduced the number of coliforms and CTX-R coliforms by 2.3 log units, while application of tertiary treatment increased the average removal of coliforms and their resistant subpopulation to 4.4 and 3.5 log units, respectively. According to Manaia (et al., 2010), large WWTPs release even 10 to 100 times more antibiotic resistant bacteria per inhabitant than smaller treatment plants. Thus, disinfection could become a recommended strategy for e.g. WWTPs >50,000 PE, where the large cost associated with implementing and maintaining the chosen disinfection system would translate into more substantial effect, e.g. taking into account large bacterial load resulting from large volume of treated wastewater, higher number of residents or more diversified inflow, including industry of hospitals, which create higher selective pressure for bacteria. Recommendation to disinfect the effluent might also be more rational in case of large WWTPs due to their greater treatment stability and being less prone to failures than smaller WWTPs, which makes the effect of the disinfection more accountable.

Based on the bacteria concentration in the effluent and designed capacity of the WWTPs, the daily load of cefotaxime-resistant coliforms released by a single, average-size WWTP ($Q \geq 10\,000$ m^3/d) could reach even 10^{11} CFU. Unfortunately, despite the global scope of the research and a significant amount of collected data, it was not possible yet to accurately predict the final load of the antibiotic-resistant population released by WWTPs to the environment, even taking into account bacterial concentrations in raw wastewater, regional antibiotic consumption or ambient temperature, which were suspected of playing a role in the emergence and spread of antibiotic resistance, or in bacterial population dynamics. Therefore, the results of **Manuscript IV** suggest the need of intensive, routine monitoring of WWTP effluents conducted on regional, national and international level, in order to identify and understand the factors governing the distribution of CTX-R coliforms in treated wastewater and to estimate the impact of their discharge on the receiving environment.

In this regard, **Manuscript IV** propose the simplified synthetic surveillance tool that can be used to investigate the prevalence of potentially ESBL-producing strains in the WWTP effluents and receiving waters. To validate the proposed monitoring setup, the synergistic approach was applied: bacterial cultivation was combined with DNA-based identification of isolated strains to test the impact and justify

the change of incubation temperature. Previously existing standard *E. coli* monitoring procedure did not provide information on the prevalence of antibiotic resistance among bacteria. The modification of the procedure, proposed in **Manuscript IV** resulted in development of universal, relatively simple, low-cost and widely applicable cultivation-based method that can provide insight into the potential contribution of wastewater to the spread of antibiotic resistance, which is important from an environmental and epidemiological perspective (Berendonk et al., 2015; Manaia, 2017). Additional advantage of the proposed solution is that it can be adopted with only minor modification to the existing regional, national, European or international monitoring programs, using previously applied research equipment, with minimal added costs.

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2. **Kalinowska A**., Łuczkiewicz A., "*Pomeranian wastewater treatment plants as hot-spots of antibiotic resistance: the impact on the coastal waters of the Baltic Sea"* Linnaeus Eco-Tech 2020 12th International Conference on the Establishment of Cooperation between Companies and Institutions in the Nordic Countries, the Baltic Sea Region and the World, 23-25.11.2020, Kalmar, Sweden

6 SUMMARY, FINAL CONCLUSIONS AND RECOMMENDATIONS

Presented thesis aimed to characterize the wastewater composition by means of a wide range of chemical and microbiological parameters, in order to investigate the impact of the wastewater discharge to the surface waters in various geographical locations. A variety of analytical techniques were applied to create a synergistic advantage of complementary methods: chemical analysis supported microbial data in **Manuscripts I, II and III**, while in **Manuscript IV** next generation sequencing complemented cultivation, enabling the development and validation of simplified monitoring procedure.

The results provided data for the discussion on the possible environmental hazards and treatment technologies tailored to the study sites, with special attention given to polar areas (**Manuscript I and II**). Because of uniqueness and relatively pristine character, this region can serve as environmental laboratory to observe human-induced changes, e.g. related to wastewater discharge. This is especially important also in context of ongoing climate change and global warming, being particularly visible in the Arctic.

Due to the universality of this subject, the wastewater characteristic and its impact on the environment have been further investigated from the global perspective, also in the areas subjected to large anthropogenic pressure (**Manuscript III and IV**). Novel type of wastewater-associated hazard – dissemination of antibiotic resistance among bacteria – has been taken into account. Therefore, the existing procedure for monitoring the presence of fecal coliforms has been modified in order to investigate the prevalence of potentially ESBL-producing strains in the WWTP effluents and receiving waters (**Manuscript IV**).

According to the obtained results it can be stated that:

1) In polar areas, there is lack of baseline conditions in water/wastewater sector, which are necessary to evaluate the environmental changes resulting from increasing human activity;

2) Growing number of inhabitants and tourists in the Arctic increases the amount of generated wastewater, which, although usually neglected, is becoming a crucial part of the anthropogenic impact;

3) Despite meeting discharge quality requirements, WWTPs based on biological treatment still discharge bacteria associated with activated sludge and human gut into receiving waters;

4) The functional potential and structural diversity of the microbial community in treated wastewater undergoes changes throughout the year;

5) The elevated abundance of prokaryotic cells in WWTP effluent, including functional nitrification/denitrification organisms and filamentous, bulking or human-gut bacteria, was related to the seasonal decrease in treatment efficiency;

6) Sampling points under the direct inflow of wastewater were characterized by higher values of total cell number (TCN), prokaryotic biomass (PB), average cell volume (ACV) and live cells percentage (LD), when compared to reference points not subjected to wastewater release (on average 2 times higher TCN and PB, slightly elevated ACV and LD);

7) WWTP effluents contained different functional microorganisms, when compared to natural waters, e.g. treated wastewater contained *Nitrosomonas* and *Nitrospira*, while in natural waters nitrifying bacteria were represented by *Nitrospumilus* and *Nitrospina*;

8) Even if the presence of wastewater-derived bacteria in the receiver was confirmed, their further activity or adaptation in the environment is largely unknown;

9) The volume of the discharged wastewater, together with the size, dynamics and the ecological status of the receiving waters are important factors when evaluating the impact of the WWTP effluent of the recipient;

10) Novel pollutants such as resistant bacteria, resistance genes and integrons were present in untreated and treated wastewater generated in both small Arctic settlements and large-scale WWTPs catchments;

11) Those indicators of antibiotic resistance were also found in the wastewater recipients;

12) Treated wastewater from the Polish Polar Station (up to Q=3.6 m^3/d) released to the Arctic environment up to 7×10^6 *Enterococcus* CFU per day, including bacteria exhibiting clinical resistance to e.g. nitrofurantoin (14.3% of tested *E. faecalis* isolates from treated effluent);

13) WWTP effluent from the Polish Polar Station contained also presumptive non-wild type *Enterococcus* isolates, possessing acquired or mutational resistance mechanisms to nitrofurantoin, moxifloxacin and erythromycin (MIC above ECOFF values);

14) To monitor emerging resistance patterns, a modification of the existing culture-based procedure was suggested and validated, in order to be used instead of advanced MIC-based methods;

15) The proposed supplementation of standard cultivation medium with cefotaxime provided the insight into the beta-lactam resistance among coliforms;

16) The global studies showed that despite the high rate of bacteria reduction (99% by secondary and 99.99% by tertiary treatment), an average large-scale WWTP ($Q \geq 10\ 000\ m^3/d$) can still release up to 10^{11} CFU of the cefotaxime resistant coliforms to the environment per day;

17) Nevertheless, the exact load of resistant bacteria released with treated wastewater was difficult to predict, even taking into account the available data on drug consumption or concentration of resistant bacterial fraction coming to the WWTP with untreated wastewater;

18) The continuous discharge of treated wastewater containing antibiotic resistant bacteria suggests the need of routine monitoring of WWTP effluents and their recipients on regional, national and international level, in order to assess the dissemination of antibiotic resistance;

19) The proposed procedure for beta-lactam resistance investigation in coliforms can be adopted worldwide with low requirements for professional expertise and minimal additional cost;

20) Therefore, the method is appropriate for the aforementioned baseline monitoring and can be used to investigate the emerging resistance levels in treated wastewater and receiving waters;

According to the results, it can be concluded that the holistic approach and the use of diverse analytical methods helped to characterize the wastewater, especially in terms of frequently overlooked microbial parameters: the quantity, viability and composition of prokaryotic community, as well as the presence of functional nitrification/denitrification bacteria and genes, or prevalence of antibiotic resistance among human-related fecal indicators. According to author's best knowledge, this was one of the first studies quantifying the nitrogen-cycling genes in the WWTP effluent and its marine recipient. Multivariate analysis enabled the identification of the general trends in microbial community of treated wastewater, shown to be associated with seasonal deterioration of treatment efficiency. Yet, there still exists a knowledge gap on the fate of wastewater-derived bacteria in the environment: their survival, activity or adaptation.

Combining a variety of tools and techniques provided a synergistic benefit, supporting the strengths and balancing the drawbacks of the applied methods. This can be valuable for the pilot studies and research purposes, when the subject and correlations are not fully recognized. Thus, the holistic approach was justified when analysing the impact of wastewater discharge on the receiving waters.

Combination of different methods was also used to validate the proposed new monitoring procedure. Nevertheless, the environmental monitoring itself should be as universal, standard and simple as possible (e.g. only as a minor modification of existing, everyday used methods). Minimal requirements for man-hours, professional expertise, materials or additional infrastructure will contribute to the success of applying surveillance all over the world. It is also to be noted that the global monitoring aims mostly for the general

trends. If alarming, they can be analysed in depth and verified with advanced methods that require specialized knowledge or expensive laboratory. In this study, the proposed surveillance procedure addressed emerging resistance levels in treated wastewater and receiving waters. The data obtained with this tool could be used to understand the factors and mechanisms governing the maintenance and selection of antibiotic resistance in wastewater treatment systems, thereby improving the risk assessment and estimating the safe allowable levels of ARG and ARB in WWTP effluents that would not pose risks for human and environmental health.

Despite already reported high effectiveness of wastewater treatment processes, new threats associated with novel pollutants constantly arise. There is no existing technology, that would remove all the contaminants and eliminate all the hazards. The mitigation strategy proposed in this thesis addressed systemic monitoring of pharmaceutical consumption or usage of personal care products and replacing them with environmentally friendly alternatives, what is in line with the European Union strategy to limit and prevent pollution effectively at sources. In case these measures are inefficient due to the population increase or any other reason, then the application of the advanced end-of-pipe, locally tailored solutions, targeting precisely identified risk would be the final barrier for the pollution.

It is worth to note that this PhD thesis is also in line with the proposed revision of the Urban Wastewater Treatment Directive, which recognizes the risks associated with small (below 2 000 p.e.) agglomerations, particularly discharge of emerging pollutants or fecal indicators. It also emphasizes the identification of novel, upcoming threats and using the wastewater parameters as a support for public health sector. New Directive also sets a goal to align the wastewater sector to the objectives of the Green Deal: by steering it towards Energy neutrality as a contribution to Climate Neutrality, and by supporting its necessary transition towards Circular Economy and Zero Pollution. All the above demonstrate the directions for wastewater treatment development and indicate that it has to adapt to the future challenges. This thesis shed the light on some of the wastewater-related microbial hazards. The proposed monitoring strategy will help to assess whether the modifications in the wastewater sector would be sufficient and will contribute significantly to improve the environment conditions, also in regards to novel threats.

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Heavy Metals in a High Arctic Fiord and Their Introduction with the Wastewater: A Case Study of Adventfjorden-Longyearbyen System, Svalbard

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Abstract: Longyearbyen is the largest settlement on Svalbard archipelago, with 2400 permanent residents and approximately 150,000 tourists visiting every year. The city annually releases approximately 285,000 m^3 of untreated wastewater to the nearby Adventfjorden. To date, the environmental impact of this continuous input has been studied mainly regarding the sediments and benthic fauna in the fiord. Here, we present results from a study of raw wastewater entering Adventfjorden as well as heavy metals concentrations in the water column within the fjord itself. Two surveys were carried out in summer and autumn season 2018, to establish physical and chemical properties of water at various locations. Trace elements (V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Hg, As, Cd, Pb, U), total suspended solids (TSS) and total organic carbon (TOC) were measured. Our results show that Longyearbyen's raw wastewater introduces low concentrations of heavy metals to the fiord, but due to the growing number of inhabitants and tourists, it should be monitored to avoid degradation of Adventfjorden ecosystem

Keywords: High Arctic marine environment; wastewater discharge; heavy metals; ICP-MS; water pollution in the Arctic

1. Introduction

Svalbard archipelago is located in the High Arctic between 76.50–80.80° N and 10–34° E [1]. Despite its large area, the density of human population is very low (circa 0.04 person/km^2). This is because the majority of the population is concentrated in two major towns in the middle of the Spitsbergen island, Longyearbyen and Barentsburg, with a few small satellite research centers along the west

coast. Both Longyearbyen and Barentsburg were established as mining towns in the beginning of the 19th century—therefore, throughout the years, coal mining left a distinct anthropogenic impact on the neighboring environment. Even though today, mining activity on Spitsbergen have significantly decreased (coal production dropped from over 4 million tons in 2007 to around 1 million tons in 2015) [2] and has been largely replaced by the ever-growing tourism industry, interest in pollutants pathways (via air, water and ground) and their fate in the Arctic environment is still rising. For example, in Longyearbyen, which is the administrative centre and the largest settlement of Svalbard, the wastewater is collected and directed untreated to the nearby Adventfjorden. Therefore, the wastewater discharge can be regarded as an important source of contaminants, such as nitrogen, phosphorus, organic matter, as well as human-related bacteria. Untreated wastewater may also contain heavy metals, which not only can have a deteriorating effect on the food web, but also may undergo bioaccumulation and biomagnification starting at the bottom of the food chain, with the ice algae or phytoplankton [3]. Furthermore, heavy metals also act as a stress factors, inducing, e.g., plasmid-related resistance against heavy metals among bacterial communities [1,4–6] and/or inhibiting microbiological processes [7].

Despite the above, the concentrations of heavy metals in wastewater in polar regions as well as their fate in marine environment have been investigated in only a few studies of marine sediments in Antarctica [8–10]. This lack of comprehensive research is partially due to the complexity/multitude of heavy metal sources that contribute to their concentrations in the High Arctic. Allochthonous sources include long-range atmospheric transport, volcanic eruptions or even wildfires [11–14], while autochthonous emission centers are commonly associated with coal mining and acid mine drainage, coal burning, road and air traffic as well as the energy sector [13]. Despite the scientific interest in heavy metals in the Arctic environment, to date, research has been focused mainly on soil [12,13], lake sediments [12,15], snow and glacier ice [12,16–18] as well as plants [19], fish and other animals [12,20–24].

In domestic wastewater, the household sources of heavy metals can be: laundry detergents and other personal care products, medicines, food, kitchen utensils and piping, but also tap water (Table 1). It is of special concern in Arctic areas; knowing the effects of anthropogenic pollution is a pressing matter since the population of Longyearbyen is continuously growing: from 25 people in 1906 up to 2400 inhabitants in 2019. Similarly, the number of tourists has doubled over the past 10 years, and in 2018, it reached about 150,000 visitors per year [2]. Increasing activity in this area is directly connected to rising untreated wastewater release to the fiord each year.

Therefore, here, we present a coupled wastewater-fjord study of trace elements that was performed in the vicinity of Longyearbyen. The focus was given to the concentration of heavy metals in the wastewater as well as their seasonal and spatial distribution in the wastewater receiver—the Adventfjorden.

Table 1. Typical sources of heavy metals in the domestic wastewater.

Domestic Sources of Heavy Metals in Wastewater	Cu	Zn	Pb	Cr	Ni	Cd	Hg	Mn	Fe	Co	As	Reference
Household contribution to heavy metals content in the typical municipal wastewater	27–100%	30–46%	0.9–15%	2.4–15%	9–61%	20–60%		8%	9%		21%	[25–29]
Plumbing	✓	✓	✓	✓	✓							[25–27,30,31]
Laundry detergents	✓	✓	✓	✓	✓	✓	✓				✓	[26,27,31–34]
Tap water	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	[26,27,31,35–37]
Kitchen utensils			✓	✓	✓	✓					✓	[25–27,38–44]
Food	✓	✓	✓	✓	✓	✓	✓	✓			✓	[26,37,45]
Cosmetics (PCP—personal care products): toothpaste, deodorant, shampoo		✓		✓							✓	[26]
Medicines											✓	[25]
Feces					✓			✓				[29,46–48]
Amalgam	✓	✓					✓					[26]
Artist paint						✓						[26]

2. Materials and Methods

2.1. Study Area

The study was conducted in the central part of West Spitsbergen island (Svalbard archipelago) in the vicinity of the largest settlement on the island, Longyearbyen (Figure 1), which discharges untreated wastewater (about 285,000 m^3/year) to the nearby Adventfjorden. Adventfjorden is a part of the biggest fjord on the west coast of Spitsbergen, Isfjorden. Its wide and deep mouth facilitates the water exchange with the central part of Isfjorden due to tidal pumping and wind-driven surface currents [49].

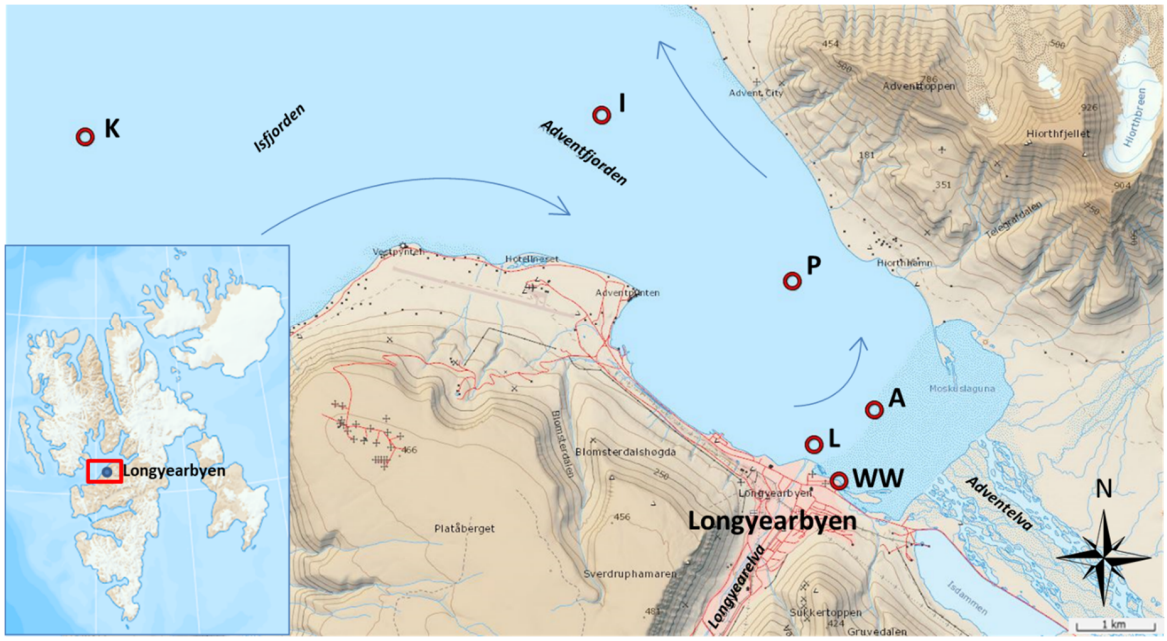

Figure 1. Location of the study in Adventfjorden, Isfjorden. The circles represent sampling points (K = Karlskrona depth, reference point in Isfjorden; I = IsA station, reference point in Adventfjorden; P = wastewater pipe outlet to Adventfjorden, A = Adventelva outlet to Adventfjorden; L = Longyearelva outlet to Adventfjorden; WW= raw wastewater pumping station). The arrows represent dominant water current in Adventfjorden (modified from toposvalbard.no).

The largest contributors of freshwater into Adventfjorden are two, predominantly glacier-fed rivers: Adventelva and Longyearelva. Thus, they discharge large amount of freshwater (between 0.4 and 0.8 m/year/km^2, Nowak pers. comm) and suspended solids to the fiord (up to 1 g/L in the melting season, mostly silt) [50,51]. Rivers can be also enriched in trace elements due to the presence of mining sites around Longyearbyen. They reach the maximum flow rate in the summer months, which significantly influences the physicochemical conditions in the internal part of the fiord (near river mouth—points L and A), while in autumn, their inflow ceases.

2.2. Sampling

Sampling points in Adventfjorden (Figure 1) were selected to reflect the inflow of wastewater to the fiord (sampling point P) and the impact of two glacial rivers: Adventelva and Longyearelva (sampling points: A and L, respectively). In addition, two reference points were chosen to include dominant water current in Adventfjorden (Figure 1): station K (Karlskrona depth) located in the central part of Isfjorden, and station I, located inside Adventfjorden. Station K was assumed not to be impacted by the wastewater release, while station I could receive some of the pollution due to the water circulation in the fiord. Water samples in Adventfjorden were collected with a 10 L Niskin bottle at

the surface (0–1 m) and bottom (~5–10 m above sea bed) of the water column. To minimize the risk of contamination, samples were collected using nitrile gloves and bottles were rinsed three times with sampled water and then filled completely without air bubbles. Simultaneously, untreated wastewater was collected prior discharge to the receiver—from the last pumping station on the sewer system (sampling point WW, Figure 1). All the samples were kept frozen until further analysis. Samples were collected twice: in summer (July) and in autumn (October) 2018. Preceding sampling, the average daily temperatures varied around 5–10 °C in July and from −12 °C to 5 °C in October. Precipitation did not exceed 0.4 mm/week prior to both of the sampling campaigns.

2.3. Sample Analysis

Total organic carbon (TOC) concentration was determined by a TOC-VCSH/CSN Analyser (SHIMADZU, Japan) using the catalytic combustion method with non-dispersive infrared detection (NDIR). Total suspended solids (TSS) were determined in duplicates by the membrane filtration method according to the Standard Methods [52]. The concentrations of metals: arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), nickel (Ni), uranium (U), vanadium (V) and zinc (Zn) in water and wastewater samples were determined by inductively coupled plasma mass spectrometry, Thermo XSERIES 2 ICP-MS (Thermo Fischer Scientific, Germany). The certified reference material EnviroMAT Ground Water (Low ES-L-2 and High ES-H-2) was used to validate the method. Trace element concentration CVs of the obtained triplicate results ranged from 0.5% to 3.0%. Measurement range (MR), limit of detection (LOD) and limit of quantification (LOQ) are specified in Table 2.

Table 2. Analytical techniques employed to determine the analytes, together with selected validation parameters characterizing the analytical methods used.

Analyte/Parameter	Measurement Instrumentation	MR	LOD	LOQ	Unit
Cd	Inductively Coupled Plasma Mass Spectrometer Thermo XSERIES 2 ICP-MS (Thermo Fischer Scientific)	0.01–500	0.01	0.03	$\mu g\ L^{-1}$
Co		0.01–500	0.01	0.03	
V,Cr,Mn,Ni,Cu,Zn,As,Pb,U		0.10–500	0.10	0.30	
Fe		0.15–500	0.15	0.45	
Total Organic Carbon (TOC)	Total Organic Carbon Analyzer TOC-V CSH (Shimadzu)	0.03–200	0.03	0.10	$mg\ L^{-1}$

MR—measurement range; LOD—limit of detection; LOQ—limit of quantification.

2.4. Data Analysis

To detect pair-wise relationships among the metals, salinity, TOC and TSS concentration in the investigated water samples, Pearson's correlation coefficients (r) were calculated using Excel 2010. Statistical significance of correlation coefficients was assessed at a significance level of $p < 0.05$. The statistical significance of correlations was verified using the Student's *t*-test.

3. Results

3.1. Heavy Metals Concentrations in the Wastewater and Adventfjorden

Wastewater generated in Longyearbyen contained higher concentrations of all heavy metals than the recipient, with the exception of U and Cd (Table 3). The highest concentrations were noted in the case of iron (226 ± 79 µg/L) and manganese (176 ± 62 µg/L) and the lowest were in case of Pb (1.12 ± 0.53 µg/L), U (0.11 ± 0.02 µg/L) and Cd (0.03 ± 0.01 µg/L) (Table 4). The order of heavy metals abundance in the untreated wastewater collected prior discharge to the Adventfjorden (sampling point WW) was as follows: Fe > Mn >> Ni ≥ Zn > Cu ≥ As ≥ V ≥ Co ≥ Cr ≥ Pb > U > Cd.

Table 3. Heavy metals concentrations in Longyearbyen's water supply reservoirs, potable water system, wastewater and its marine recipient, Adventfjorden.

Heavy Metal	Drinking Water Reservoirs				Potable Water System		Limit Values for Potable Water	Wastewater		Wastewater Recipient				Typical Values Found in the Marine Waters	
	Isdammen		Gruvedalen												
	Concentration (μg/L)														
	Min	Max	Min	Max	Min	Max		Min	Max	Mean	Sd	Min	Max	Min	Max
As	0.03	0.22	0.05	0.25	0.03	0.1	10	1.57	2.55	1.25	0.41	0.42	2.01	1	2
Cd	0.02	1.0	0.07	0.17	0.01	1.0	5	0.02	0.03	0.02	0.01	0.01	0.04	0.01	0.07
Co	0.05	2.96	0.64	12.68	no data	no data	-	1.55	1.59	0.09	0.08	0.02	0.32	0.003	7.7
Cr	0.76	4.25	0.1	0.57	no data	no data	50	1.48	1.51	0.19	0.06	0.1	0.4	0.31	0.65
Cu	0.1	7.94	0.4	9.3	no data	no data	1000	1.69	2.73	0.42	0.32	0.15	1.58	0.1	1.07
Fe	5.0	1650	10.0	811	6.0	98	200	170	282	2.09	1.14	0.94	4.86	0.32	1.29
Mn	4.0	660	29.0	253	5.0	510	50	132	220	1.27	1.12	0.14	3.88	0.01	1.1
Ni	0.1	6.3	3.78	30.9	no data	no data	20	11.9	13.1	0.37	0.24	0.06	1.01	0.1	0.3
Pb	0.93	1.32	0.04	0.27	no data	no data	10	0.74	1.49	0.11	0.01	0.09	0.13	0.01	0.05
U	no data	no data	no data	no data	no data	no data	30	0.1	0.12	0.44	0.04	0.32	0.53	1.5	4.7
V	<LOD	0.03	<LOD	0.06	no data	no data	-	1.47	2.61	0.63	0.21	0.25	0.91	0.1	1
Zn	0.9	29.7	4.79	77.44	no data	no data	-	3.9	12.3	1.45	0.76	0.56	3.35	0.07	2.14
Reference	[53]		[53]		[53]		[54]	This survey		This survey				[3,55–57]	

Table 4. Concentrations of heavy metals detected in wastewater from Longyearbyen, polar scientific stations and typically met in the domestic, urban and municipal wastewater.

Research Area	Mean ± SD/Median	Concentration (μg/L)											Reference
		Cu	Zn	Pb	Cr	Ni	Cd	Mn	Fe	As	Co	V	
untreated wastewater, Longyearbyen, Svalbard	mean	2.21	8.10	1.12	1.50	12.51	0.03	176	226	2.06	1.57	2.04	This survey
	SD	0.74	5.88	0.53	0.02	0.83	0.01	62	79	0.69	0.03	0.81	
untreated wastewater, McMurdo Antarctic Station	mean	564	513	376	32	31	3.41	13.1	1646		3.33		[8]
	SD	1073	230	1169	16.8	14	2.92	8.0	1205		0.98		
untreated wastewater, Davis Station, Antarctica	mean	870	1210	37	20	42	4.2	176					[9]
	SD	499	939	8	22			146					
Untreated wastewater, Henryk Arctowski Polish Antarctic Station	mean	4.27	37.3	0.48	4.44	23.30	0.45	28.9	428		1.68		[58]
Domestic wastewater, residential area, Stockholm	mean	78	150	3.6	4	6.2	0.23						[26]
Domestic wastewater, Ostrava, Czech Republic	median	19.5	167	5.5	2.546	3.5	1.0	77.0	872	0.6			[25]
Wastewater influent to WWTP Kravare, Czech Republic	median	21.3	181	5.0	2.761	4.0	1.0	69.0	963	0.5			
Urban wastewater, inflow to WWTP Ostrava, Czech Republic	mean	35.0	230.0	17.25	12.65	18.0	0.8	452	4785	1.4			
Domestic wastewater, constructed wetland influent, Zemst, Belgium	mean	7	36	2	1	3	0.1	33	45				[59]
	SD	9	28	1	0	1	0.0	8	19				
Treated wastewater, constructed wetland efluent, Zemst, Belgium	mean	3	26	2	1	3	0.1	227	28				
	SD	1	21	0	0	1	0.1	46	8				
Raw municipal wastewater, Thessaloniki, Greece	mean	79	470	39	40	770	3.3	67	480				[60]
	SD	35	140	9.4	12	200	1.1	12	87				
Raw municipal wastewater, WWTP Gdańsk	mean	125.38	439	62.58			20.59						[61]
	SD	56.17	141	27.38			14.05						
Raw municipal wastewater, WWTP Gdańsk	mean	93	300	16.00	20.60	13	0.50			4.00		6.00	[62]
	SD	30	60	12.00	16.90	7	0.30			5.00		5.00	

The concentrations of heavy metals in Adventfjorden investigated in this study followed the general order: Fe ≥ Zn ≥ Mn ≥ As ≥ V ≥ U ≥ Cu ≥ Ni ≥ Cr ≥ Pb > Co > Cd, which only to some extent follows the order represented by wastewater presented above. Moreover, in this study, the differences in heavy metals concentrations in the marine water were not as pronounced as in the wastewater. None of the metals in the recipient exceeded concentration of 5 µg/L (Figure 2). In the summer season, Pb was below detection limit (<0.10 µg/L). The concentrations of cobalt, nickel, cadmium and iron in summer season were the highest at the Adventelva outflow (sampling point A). The concentrations of vanadium in October were higher than in July and bottom samples presented higher values than surface water samples. On the contrary, manganese concentrations were on average higher in surface than bottom water samples and were the highest for surface samples in July. A similar relation with the depth was noted for Ni, Co, Fe and Zn. Fe levels also increase near the river outlets (points L and A, see Figure 2), in contrast to As. Arsenic presents higher concentrations in the bottom water samples, which is more pronounced in July.

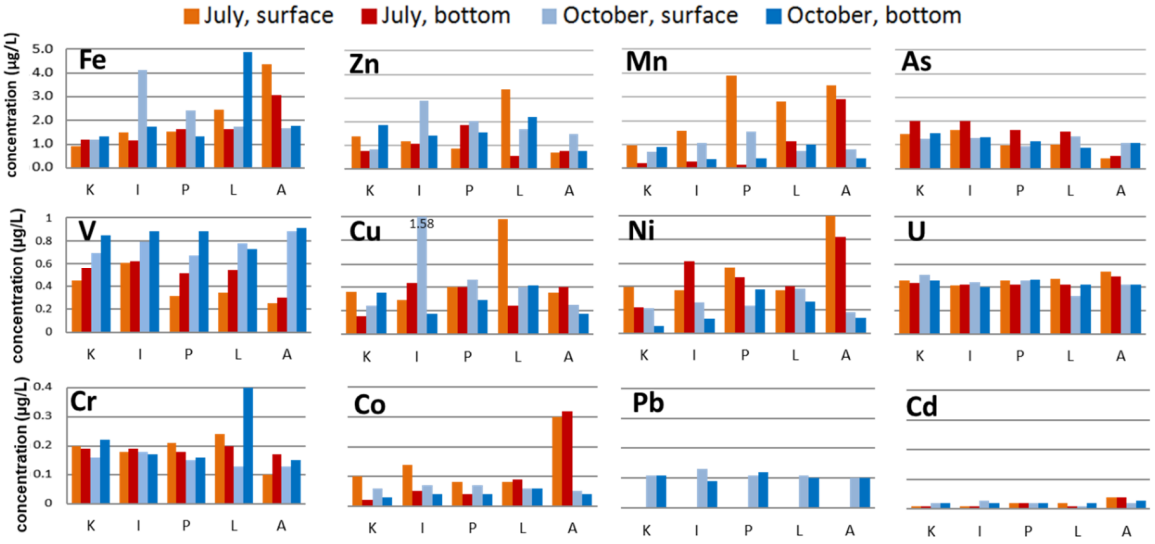

Figure 2. Concentrations of heavy metals in marine waters of Adventfjorden. The labels on the x axis refer to the name of the sampling stations (K, I, P, L, A).

The difference between heavy metals concentration in wastewater and recipient was the highest for Mn and Fe (over 100 times higher concentration in wastewater than in marine waters), Ni and Co (33 times and 18 times higher, respectively). The smallest difference between sewage and recipient was noted for As and Cd (mean value in wastewater was around 150% of the mean concentration in marine waters). U concentrations were on average higher in Adventfjorden than in the wastewater.

3.2. TSS, TOC and Correlations with Heavy Metals

In July, TSS concentration in the recipient reached values much higher than in October (up to 835.11 mg/L versus 11.40 mg/L, respectively). The highest values were recorded in July at the Adventelva outlet (point A), followed by points L and P in the inner Adventfiorden basin (53.60–62.2 mg/L in the surface water samples and 16.1–29.5 mg/L in the bottom layer). The lowest TSS was noted for outer stations K and I (2.47–10.8 mg/L). In October, when the river discharge into the fiord decreases, TSS concentration did not exceed 12 mg/L at all stations (Figure 3).

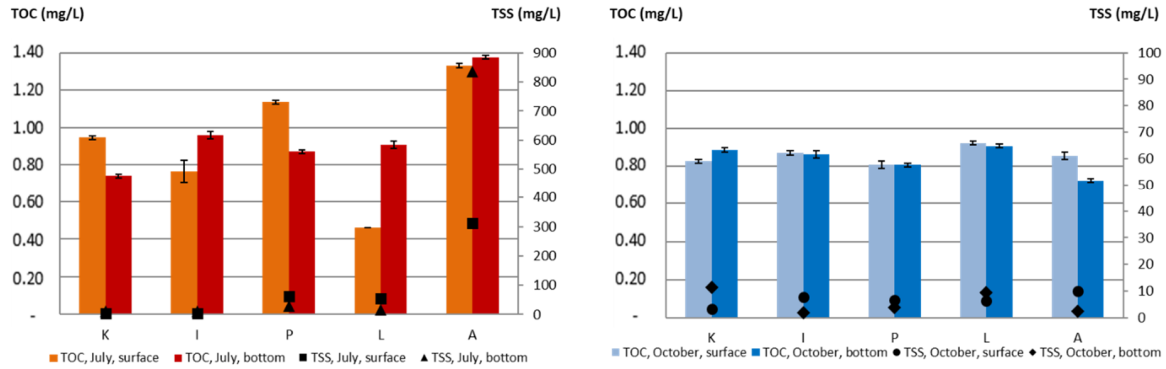

Figure 3. Total organic carbon and suspended solids concentrations in the recipient in July (**left**) and October (**right**). The bars refer to TOC values, the black symbols to TSS. Note different scales for TSS. The labels on the x axis refer to the name of the sampling stations (K, I, P, L, A).

Total organic carbon (TOC) concentrations were around 40 times higher in wastewater samples (sampling point WW), than in the recipient (mean of 39 mg/L in wastewater versus mean 0.9 mg/L in marine waters). TOC values in Adventfjorden oscillated from 0.85 ± 0.06 mg/L in October to 0.95 ± 0.28 mg/L in July (Figure 3) and the highest values were found and wastewater discharge pipe and at the mouth of Adventelva river in July (sampling points P and A, respectively). In October, no significant correlation was found between TSS and TOC, even though the correlations in July and for the whole set of data were strong (0.69 mg/L and 0.71 mg/L, respectively, see Table 5).

Table 5. Values of Pearson correlation coefficient (r) for total concentrations of investigated elements (selected metals nonmetals and total organic carbon concentration). Strong and very strong correlations ($0.6 < |r| \leq 1$) are given in bold. Grey background emphasize very strong correlations ($0.8 < |r| \leq 1$). Values of Pearson's correlation coefficients in the range ($0.6 < |r| \leq 1$) are statistically significant at $p < 0.05$.

Analyzed Parameter	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Cd	Pb	U	TOC	TSS
V	1													
Cr	0.832	1.000												
Mn	0.896	0.958	1.000											
Fe	0.721	0.955	0.870	1.000										
Co	0.774	0.968	0.950	0.957	1.000									
Ni	0.835	0.984	0.977	0.952	0.990	1.000								
Cu	0.592	0.833	0.744	0.876	0.827	0.820	1.000							
Zn	0.504	0.801	0.638	0.907	0.779	0.764	0.908	1.000						
As	0.392	0.487	0.384	0.545	0.399	0.453	0.443	0.538	1.000					
Cd	0.006	0.173	0.145	0.273	0.311	0.218	0.349	0.296	−0.430	1.000				
Pb	0.706	0.913	0.807	0.985	0.907	0.901	0.873	0.936	0.544	0.296	1.000			
U	−0.841	−0.914	−0.876	−0.897	−0.870	−0.905	−0.758	−0.767	−0.622	0.021	−0.878	1.000		
TOC	0.824	0.988	0.958	0.975	0.987	0.995	0.841	0.813	0.494	0.223	0.937	−0.919	1.000	
TSS	−0.536	−0.154	0.541	0.361	0.862	0.669	−0.006	−0.270	−0.565	0.671	−0.334	0.457	0.707	1.000

4. Discussion

4.1. Heavy Metals in the Wastewater

Wastewater generated in Longyearbyen contained higher heavy metals concentrations than the Advenfjorden, thus, it can be considered as a point source of pollution. Apart from nickel and manganese, heavy metals concentrations in wastewater from Longyearbyen were lower than in typical municipal wastewater [25,26,59,61] and wastewater generated at the Antarctic stations [8,9,58], see Table 4. The higher concentrations in the municipal wastewater should be attributed to the industrial contribution, while at Antarctic stations the main explanation may be the lack of dilution, as those settlements usually operate at a minimum water consumption, with a less efficient potable water supply system than in Longyearbyen.

The order of heavy metals abundance in the untreated wastewater (Fe > Mn >> Ni ≥ Zn > Cu ≥ As ≥ V ≥ Co ≥ Cr ≥ Pb > U > Cd) is mostly in agreement with the relative abundance reported by Chipasa [61] and Karvelas [60] in municipal wastewater, apart from Ni and Mn abundances, which are higher in case of Longyearbyen wastewater.

In domestic wastewater, trace elements can originate from toilet paper, detergents, medicines cosmetics and other personal care products [25–27,29], see Table 1. Moreover, food leftovers can contain trace elements—note that in Longyearbyen, they are milled in sinks and directed to the sewer. Sörme and Laderkvist [26] indicated household emissions, such as food and artistic paints, as the second largest contributor of Cd in the wastewater. It has been estimated that approximately 100 tonnes of food is discharged to the fjord every year. Food waste constitutes about 0.4 m^3 daily, which can be of a significant importance with regard to local organic load and the supply of certain heavy metals [63].

Plumbing material, especially copper piping, which is also used in the Longyearbyen water supply system, was pointed out by Comber and Gunn [27] and Sörme and Lagerkvist [26] as an additional source of trace elements (mainly Cu, Pb and Zn) in wastewater. Taps, heat exchangers in housing, iron valves in the pumping stations and slightly acidic pH may also contribute to heavy metals load in Longyerbyen water and then wastewater. Indirectly, it is confirmed by a large corrosion on the metal pipes that has been reported in town (Kjersti Olsen Ingerø, Longyearbyen Lokalstyre, pers. comm.).

In Longyearbyen, the drinking water system is supplied by two meltwater and rain fed reservoirs in Gruvedalen and Isdammen watershed. Nowak and Hodson [64] indicated the influence of acid mine drainage on the quality of drinking water sources, especially at the beginning and end of the summer. In spring, heavy metals are delivered by melting snowpack and can be leached from the underlying mine waste rock. At the end of the summer, the surface runoff is lower and the rock-water contact is prolonged, also resulting in higher trace elements concentrations. Heavy rainfalls during the year may deliver contamination from the hillslopes, but also dilute them. In Longyearbyen's drinking water reservoirs (Isdammen and Gruvedalen) concentrations of Mn, Fe, Cr, Co, Cd, Cu, Zn and Ni were noted to be higher than those in the wastewater (Table 3). For example, in Isdammen reservoir over 70% of the analyses in years 2005–2017 revealed manganese concentrations higher than allowed for drinking water [53], indicating the need of pre-treatment. In this case, drinking water may contribute to a significant share of heavy metals in the wastewater, especially Mn, Fe, Cr, Co, Cd, Cu, Zn, Ni, Pb (Table 3).

4.2. Heavy Metals in the Adventfjorden

In the summer, the highest concentrations of Co, Ni, Cd and Fe were noted at the Adventelva outflow (sampling point A), which suggests the impact of the river. This is supported by Bazzano et al. [65], who found that natural input from the glaciers is the major source for Co, Fe and V in another fjord system in Svalbard, Kongsfjorden. In case of vanadium, however, this trend in our data cannot be seen. This suggests that other factors rather than river discharge can play a more significant role in the distribution of vanadium concentrations. Manganese and cobalt seem to depend on the sampling depth in the fiord, but also reveal higher concentrations at the river mouth

in the summer (similarly to Fe), which suggests the contribution of freshwater to discharge these metals into the fiord. Additionally, the physical mixing of the fresh water with seawater masses influences the concentration of heavy metals, which was confirmed for dissolved arsenic in estuarine waters [66–70]. The increase of As concentrations with the increasing salinity (freshwater versus seawater) observed in our dataset was also recorded in Krka Estuary [71]. The lowest As values in our data was observed at the stations with the greatest freshwater influence in the period when river discharge is the highest. Higher concentrations in the bottom water masses are especially visible in data from the summer (Figure 2) when the stratification in the fiord is the strongest. Separation of water masses slows down mixing and therefore, restricts vertical migration of pollutants that are not bound to solid particles and therefore, are not prone to settling. According to Bazzano et al. [65], Cr, Cu, Mn, Ni, Pb and Zn in the Arctic fiord were mainly introduced with the inflow of Atlantic waters. This may be reflected by the higher concentrations of Cr, Cu and Zn observed at Longyearelva mouth (sampling point L), which is located on the way of general water current in Adventfiorden (see Figure 1).

The sources of heavy metals in the fiord, apart from wastewater discharge, can also be associated with other human activities, which makes tracking of pollution origin in the fiord a complex task. For instance, allochthonous sources of heavy metals Longyearbyen may include ships and airplanes, which may contribute to heavy metals emission into the environment. In addition, old waste dumping sites have been frequently mentioned as a heavy metals pollution source in the vicinity of human settlements in polar regions [8]. In the case of Longyearbyen, the dumpsite at the seashore was closed in 1990 [63] and moved further from the fiord; however, the risk of leaching heavy metals from the old dumping site or coal mining constructions remnants still exists. In the case of Cd, however, there is little evidence that its concentration in the marine waters has been impacted by human activities [3,72], which is in contrary to nickel. Its relatively high concentration in biota around Longyearbyen has been attributed to the mining activity in the area [35]. As mentioned above, some contaminants could be potentially delivered into Adventfjorden via river discharge.

4.3. Correlations with TSS and TOC

Total suspended solids values correlated with the Adventelva and Longyearelva inflow, as they introduce a large amount of coarse silt. This relation has also been noted in other studies in the area [50,73]. In contrary to the wastewater, TSS originated from rivers is mainly in mineral form [Kalinowska, unpublished data]. This may be of importance, as heavy metals usually show correlations with the organic matter content [74]. Wastewater is the main contributor of TOC and heavy metals to the recipient. Suspended solids, which are mainly of the glacial river origin, do not contribute strongly to the heavy metals input to the fiord. The influence of the surface runoff or remobilization of heavy metals from the sediments has not, however, been studied.

5. Conclusions

We found that untreated wastewater discharged by Longyearbyen to the nearby Adventfjorden delivers heavy metals in concentrations similar or lower to typical domestic wastewater, also those from polar scientific stations. Despite the low pollutants concentration, wastewater can be considered a local pollution point source, as it contains slightly higher concentrations of heavy metals that the waters of its marine recipient.

No spatial or seasonal pattern in distribution in the recipient was found for all the elements analyzed. Nevertheless, some patterns, specific for individual metals, were noted: concentrations of manganese, cobalt, nickel, cadmium, iron and arsenic seem to be impacted by river discharge into the fiord, which is the strongest during the summer. Rivers, however, can be influenced by the vicinity of former or still existing mining sites. Chromium, copper and zinc concentrations can partly depend on Atlantic waters inflow into the Advenfjorden. Taking into account the relatively small volume of wastewater introduced to the Adventfjorden-Isfjorden system and dynamic exchange with water outside the fiord, we conclude that the wastewater is diluted and the concentrations of heavy metals in

Adventfjorden are influenced by other factors, including water masses dynamics in the fiord, oceanic currents, river inflow and mixing.

Nonetheless, further wastewater monitoring and its impact on the fiord is recommended due to the growing number of Longyearbyen's visitors and inhabitants, resulting in the increase of wastewater volume in the following years. Special attention should also be given to substances of emerging concern, such as pharmaceuticals and personal care products (PPCP), together with antibiotic resistant bacteria (ARBs) and genes (ARGs). Data collected throughout the years may serve as a basis for the local authorities to undertake the appropriate research, monitoring as well as proper prevention and mitigation action.

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Manuscript II

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The microbial community, its biochemical potential, and the antimicrobial resistance of *Enterococcus* spp. in Arctic lakes under natural and anthropogenic impact (West Spitsbergen)

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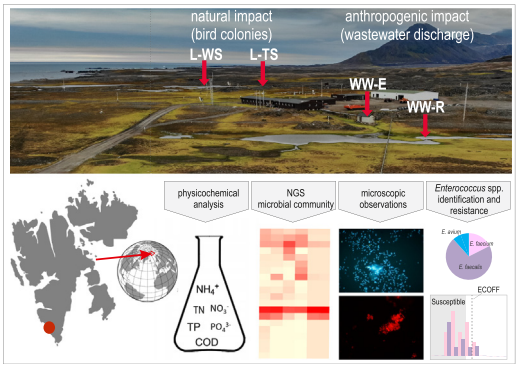
HIGHLIGHTS

- Natural and anthropogenic impact on Arctic lake ecosystems was studied.
- Nutrient-rich runoff from bird colony was retained by surrounding tundra vegetation.
- The core phyla of treated wastewater were mirrored in its recipient – Arctic lake.
- Human-related bacteria and their resistome are disseminated in Arctic lake ecosystem.
- Sustainable wastewater management is a challenge for polar human settlements.

GRAPHICAL ABSTRACT



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ABSTRACT

The sustainable management of small human communities in the Arctic is challenging. In this study, both a water supply system (Lake 1) under the natural impact of a bird-nesting area, and a wastewater receiver (Lake 2) were analysed in the vicinity of the Polish Polar Station on West Spitsbergen. Microbial community composition, abundance and activity were assessed in samples of the treated wastewater, lake water and sediments using next-generation sequencing and direct microscope counts. Special attention was given to the faecal indicator, *Enterococcus* spp., whose occurrence and antimicrobial resistance were tested in water and wastewater samples. The results indicate that Lake 1, at a tundra stream discharge (L-TS) and a water supply point (L-WS) were dominated by three phyla: *Proteobacteria* (57–58%) *Bacteroidetes* (27–29%) and *Actinobacteria* (9–10%), showing similar microbial composition up to the genus level. This suggests that nutrient-rich runoff from the bird colony was retained by surrounding tundra vegetation and reached Lake 1 at L-TS to a limited extent. Lake 2, being the wastewater recipient (WW-R), mirrors to some extent the core phyla of treated wastewater (WW-E), but in different shares. This suggests the possible washout of wastewater-related bacteria with activated sludge flocs, which was also supported by the microscopic observations. Compared to Lake 1, in WW-R an increase in all tested parameters was noted: total prokaryotic cell number, average cell volume, prokaryotic biomass and live cell percentage. The presence of *Enterococcus* spp. antibiotic resistance patterns highlight the importance of human associated microbiome and resistome dissemination via wastewater discharge. Moreover, it can be expected that

Abbreviations: L-TS, lake – tundra stream; L-WS, lake – water supply; SED-TS, sediments – tundra stream; WW-E, wastewater effluent; WW-R, wastewater recipient; SED-R, sediments - recipient; TCN, total (prokaryotic) cell number; PB, prokaryote biomass; ACV, average cell volume; SBR, sequencing batch reactor; OTU, operational taxonomic unit.

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temperature-related biochemical processes (e.g. nutrient cycling) may be accelerated by the ongoing climate change. Thus, proper wastewater treatment requires locally adapted solutions in increasingly visited and inhabited polar regions. Additionally, microbial community discharged to the environment with the treated wastewater, requires critical attention.

1. Introduction

Wastewater discharged to the surface waters can influence their physicochemical parameters (Hassan and Egozi, 2001; Igbinosa and Okoh, 2009), microbial community (Okoh et al., 2010) and lead to accumulation of chemical substances in their sediments (Marti et al., 2014). Besides the clinical settings, also wastewater is known to be an important pool of both resistance determinants and residues of antimicrobial agents (Łuczkiewicz et al., 2010; Mahfouz et al., 2018), which are introduced to sewage systems from intestinal and/or urinary tracts. Current wastewater treatment methods are insufficient in removing antimicrobial agents and they are even suspected of increasing resistance rates among bacteria due to the enhanced horizontal gene transfer (von Wintersdorff et al., 2016). This phenomenon can be promoted by high cell density and different selective pressures (sub-inhibitory concentrations of antimicrobial agents, heavy metals or other biocides and oxidative stress) occurring during wastewater treatment. However, still little is known about the development of resistance via wastewater, especially in polar areas. To date, the anthropogenic influence was regarded as negligible in these regions. Nevertheless, nowadays, the increasing number of people (inhabitants, researchers and cruise tourists) visiting the Arctic and Antarctica raises the risk of human-associated microorganisms being introduced, with unidentified consequences for the local wildlife (Hernández and González-Acuña, 2016).

Besides human beings, in polar regions other vectors of antibiotic-resistant bacteria dissemination should also be considered, e.g. migrating birds. Clinically-emerging resistance phenotypes, such as vancomycin-resistant enterococci (VRE) and extended spectrum beta-lactamase (ESBL) producing Gram-negative bacteria were isolated from glaucous gulls (Hernández and González-Acuña, 2016). These birds breed in the Arctic but are also a regular visitor to urban areas, such as city dumps and sewage outlets close to human habitats. However, only a few studies have focused on the topic (Perron et al., 2015).

A clinical approach is generally followed when defining antibiotic resistance, even in environmental research. However, it is based on the bacterial susceptibility to antimicrobial agent concentrations used during therapy (EUCAST, 2020; CLSI, 2011), and not naturally occurring in the environment. Thus, bacteria that have evolved a resistance mechanism as a response to naturally occurring antimicrobial agents (Davies, 1994; Perry et al., 2016) usually remain susceptible from the clinical point of view. Therefore in environmental studies, the resistant isolates should instead be tested using the so-called epidemiological cut-off (ECOFF) concept. ECOFF is defined based on the normal distribution of minimal inhibitory concentrations (MICs) for a given bacterial species and provides the upper MIC value for wild-type population (EUCAST, 2020). Thus, ECOFF allows wild-type species lacking the acquired and/or mutational mechanisms of resistance to be distinguished from non-wild ones with resistance mechanisms.

Besides non-indigenous microorganisms, nutrients and organic carbon too are released with wastewater to the receiver body. In polar regions it was originally thought that due to the limited number of taxa, the microbial loop there is simplified. However, currently the role of bacterioplankton in biogeochemical cycles has been recognised as crucial (Buchan et al., 2014). Additionally, changes in bacterial community structure and cell size are expected to occur as a result of climate change, higher temperatures, decreasing ice cover and higher primary production (Peter and Sommaruga, 2016; Rui et al., 2015). The knowledge of microbial behaviour and susceptibility to different stressors, including antibiotics, can increase the understanding of the links between population dynamics at different trophic levels.

Polar lakes' microbial communities are still poorly investigated (Stoeva et al., 2014) and have only recently been studied using various metagenomic methods (Górniak et al., 2016; Wang et al., 2016), mostly in terms of bacterial productivity (Adams et al., 2014) or survival of microbial populations in extreme conditions (Comeau et al., 2012). Similarly, little is known about the bacterial composition of treated wastewater and polar lakes under the impact of faecal bacteria and nutrient-rich discharge. Therefore, this study aims to fill this knowledge gap on the example of the wastewater treatment plant effluent and two Arctic lakes chosen as model areas. One is influenced by a bird nesting area (natural impact, Zielińska et al., 2016) and another receiving treated wastewater from the Polish Polar Station (anthropogenic impact). The neighbourhood of the Polish Polar Station in Hornsund, West Spitsbergen, was chosen because this area has been identified by the European Union as one of the six locations on the European continent suitable for biological and geophysical research due to its minimal transformation and environmental pollution (7th Environment Action Programme; EEAS). Additionally, Polish Polar Station wastewater treatment plant is a unique object that can serve as an example of the treated wastewater influence on polar environment. It is especially valuable in the era of increasing tourism and ongoing climate changes.

To better elucidate the ecological roles of bacterial groups, various methods were combined: metagenomic analysis (next-generation sequencing [NGS]), microscopic analysis and cultivation methods. Additionally, the identification and antimicrobial susceptibility testing of *Enterococcus* spp. was employed. This faecal indicator was chosen due to its frequency in causing multi-resistant infections and its high adaptability to harsh conditions: extreme temperatures, pH and salinity (Fisher and Phillips, 2009; Gaca and Lemos, 2019). Simultaneously, this study will help to evaluate the current biochemical properties of the microbial community in Arctic lakes and to assess the antimicrobial resistance among human-related *Enterococcus* spp., which could be used as a reference point for future research, including in the context of ongoing climate changes and increasing human impact on the polar areas. In this study, it has been hypothesized that treated wastewater discharge can significantly shape nutrient cycling, as well as taxonomic composition and antibiotic resistance of microbial community of the recipient. Apart from the anthropogenic factor, also bird migration and nesting may facilitate these changes.

2. Materials and methods

2.1. Research area and sampling

The Stanislaw Siedlecki Polish Polar Station is situated in the South Spitsbergen National Park (West Spitsbergen), at the Isbjornhamna Bay of the Hornsund Fjord (Fig. 1) since 1957, and is inhabited all year round by 10–11 crew members and up to 35 additional people (mainly researchers and technical service) during the summer season. There are no other permanent human settlements in this area.

Water and sediments were collected from two lakes near the Polish Polar Station: Lake 1 (supplier) serves as a source of potable water for the Polish Polar Station, while Lake 2 (receiver) receives treated wastewater (Fig. 1). Lake 1 was sampled at the tundra stream inflow (samples of water: L-TS, and sediments: SED-TS) and at the area of a water pumping station (water: L-WS). The tundra stream flows through a

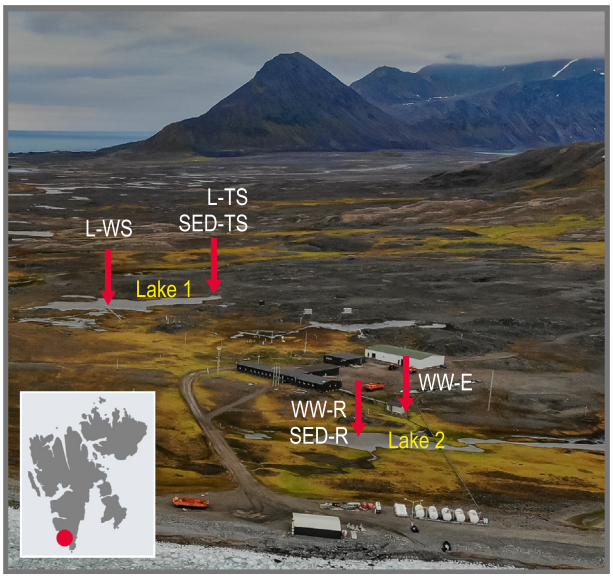


Fig. 1. Sampling area in the vicinity of Polish Polar Station in Hornsund, Spitsbergen; Lake 1 serving as a source of drinking water – sampling points: L-TS (water) and SED-TS (sediment) at tundra stream inflow and L-WS (water) at water supply area; Lake 2 serving as a receiver of treated wastewater – sampling points: WW-R (water) and SED-R (sediments) at treated wastewater discharge point; additionally, effluent from the wastewater treatment plant (WW-E) was collected; photo by Kajetan Deja.

nesting area for birds, mainly little auk colonies, which are expected to be an important source of nutrients and faecal contamination.

The treated wastewater receiver, Lake 2, was sampled at the discharge point (water sample: WW-R and sediments: SED-R). Additionally, the effluent (WW-E) of the Polish Polar Station wastewater treatment plant was also collected. Therefore, in this study the anthropogenic (human) and natural (birds) contributions to the faecal contamination of two Arctic lakes were studied.

In the Polish Polar Station, wastewater was treated mechanically by screens, and biologically by a fill-and-draw activated sludge system (two sequencing batch reactors, SBRs, 3 m^3 each, Fig. S1 – supplementary materials). To obtain high organic matter and nitrogen removal, the SBRs were working in parallel, in 180-min cycles (aerobic/anaerobic phase). Additionally, the nitrification/denitrification process was supported by the constant temperature inside the building (set at 20 °C). Excess sludge was removed from the reactors, dewatered and dried in the tanks. Note that most of the year only one SBR operates, while two SBRs are used when the number of visitors increases. For the detailed information please see the Extended characterisation of the investigated area in the supplementary materials.

Samples were collected three times, during three consecutive weeks in August 2013, and analysed in triplicates. Unless specified otherwise, the results have been presented as a mean with a standard deviation. Only samples for NGS analysis were pooled together on account of the low DNA content in a single sample.

2.2. Physicochemical parameters

Basic physical parameters (pH, temperature, electrical conductivity) were measured in situ using a pH meter combined with a temperature and conductivity meter (WTW pH/oxi 340i). Additionally, samples were stored at -20 °C and analysed at the Gdansk University of Technology. Chemical oxygen demand (COD), concentrations of nitrite nitrogen ($N\text{-}NO_2$), nitrate nitrogen ($N\text{-}NO_3$), ammonia nitrogen ($N\text{-}NH_4$), total nitrogen (TN), as well as phosphorus phosphate ($P\text{-}PO_4$) and total phosphorus (TP), were determined using spectrophotometric methods (XION 500 spectrophotometer Dr. Lange, GmbH, Germany) after transport to Poland.

2.3. Microscopic observations

2.3.1. DAPI staining

Freshwater and wastewater samples were fixed immediately after sampling with buffered formalin to a final concentration of 2% and stored at +4 °C until further analysis at the Gdansk University of Technology. Total prokaryotic cell number (TCN), average cell volume (ACV) and prokaryote biomass (PB) were determined using DAPI direct counting method (Porter and Feig, 1980). Samples were stained in 1 μg mL^{-1} final DAPI concentration for 10 min in darkness, filtered through 0.2 μm polycarbonate Whatman filters (Merck, Germany) and then rinsed twice: with 1 mL of bacterium-free distilled water and 1 mL of particle-free 80% ethanol. Filters were examined under UV light (BO-103 W high-pressure mercury burner, 330–380 nm excitation filter, 420 nm barrier filter and 400 nm dichroic mirror) with an epifluorescence microscope (Nikon Eclipse 80i) under 1000-fold magnification. Bacteria in 2 repeats of 10 fields were counted. The image analysis system of Świątecki (Świątecki, 1997) was applied. Bacterial biomass was estimated using conversion factors by Norland (Norland, 1993).

2.3.2. LIVE/DEAD staining

Staining for LIVE/DEAD analysis was performed immediately after sample collection. The fluorescent dyes SYTO9 and PI from the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA) were used in combination by mixing identical volumes of 0.1 mL of each dye and adding 0.5 mL of water sample. After dye addition, samples were incubated in darkness for approx. 30 min and filtered through 0.2 μm polycarbonate Whatman filters (Merck, Germany). Filters were kept at -20 °C until further examination. The ratio of live to dead cells was determined using an epifluorescence microscope (EX 400–440 nm, DM 455 nm, BA 470 nm and EX 450–490 nm, DM 505 nm, BA 520 nm) under 1000-fold magnification. The bacteria in 2 repeats of 10 fields were counted and the percentage of live cells was established. Live bacteria with undamaged cell membrane were seen as giving green fluorescence (ex/em: ~495 nm / ~515 nm), while damaged (dead) cells produced a bright red fluorescence (ex/em: ~495 nm / ~635 nm). The outcome of LIVE/DEAD staining (L/D) is given in percentage of live bacteria.

2.4. Isolation, identification and resistance profile of Enterococcus spp.

Enterococci were immediately cultivated from the tested water samples using the membrane filtration method (in triplicates) on 0.45 μm cellulose-acetate filters (EMD Millipore Corporation, USA) and Slanetz-Bartley *Enterococcus* selective agar (Merck, Germany). After incubation at 37 °C for 48 h (ISO 7899–2:2000) dark red or maroon colonies, assumed to represent *Enterococcus* spp., were counted and presented as colony forming units (CFU) per 100 mL. Next, for further investigations, 76 representative isolates of enterococci were taken from membranes presenting less than 20 typical colonies. For further analysis, isolates were stored in nutrient broth supplemented with 50% glycerol at -80 °C. The species identification (ID) and antimicrobial susceptibility testing (AST) of enterococci were determined by the Phoenix™ Automated Microbiology System (BD Phoenix, USA) according to the manufacturer's instructions. For ID and AST the commercially available panels (BD Phoenix, USA) were applied and *Enterococcus faecalis* ATCC 20212 was used as quality control. The antibiotic susceptibility analyses, based on the microdilution tests, were carried out against the antimicrobial agents representative for drugs important in treating human enterococcal infection (EUCAST, 2020). The identification of minimum inhibitory concentration (MIC) for certain strains was done based on epidemiological cut-off value (ECOFF) and clinical breakpoints provided by EUCAST (EUCAST, 2020). Note that the Phoenix system does not distinguish between *E. casseliflavus* and *E. gallinarum*, but it assigns the two organisms to the overlapping category: *E. casseliflavus/gallinarum*.

2.5. DNA extraction and PCR amplification of bacterial 16S rRNA gene

Water samples were filtered on polycarbonate filters (0.2 μm pore diameter, Millipore GTTP, Merck, Germany) immediately after sample collection and stored at -20 °C until the DNA extraction. Triplicates of the filtered material for each sampling point were merged for DNA extraction and considered as one sample in further taxonomic analysis. The DNA was isolated using Sherlock AX Kit (A&A Biotechnology, Poland) according to the manufacturer's instruction. The DNA concentration was determined by a Qubit 2.0 fluorometer (Invitrogen, USA).

The presence of bacterial DNA was confirmed by Real-Time PCR with SYBR Green fluorochrome, in Mx3000P thermocycler (Stratagene, USA). The following PCR conditions were used: initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of denaturation (95 °C for 15 s), annealing (58 °C for 30 s), fluorescence measurement and extension (72 °C for 30 s). For amplification of 16S rDNA fragment universal primers were applied: 1055F (5'-ATGGCTGTCGTCAGCT-3') and 1392R (5'-ACGGGCGGTGTGTAC-3') (Ferris et al., 1996). Final check on the DNA quality was done by determination of the PCR product melting curve and measuring fluorescence at temperatures from 65 °C to 95 °C. The PCR products were stored at -20 °C for sequencing.

2.6. Sequencing, taxonomic assignment and data analysis

Bacterial V3-V4 hypervariable regions of 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metagenomic Sequencing Library Protocol. The following primer pair was used for amplification: 341F (5'-CCTACGGGNGGCWGCAG-3′) and 785R (5'-GACTACHVGGGTATCTAATCC-3′). The targeted gene regions have been shown to be the most suitable for Illumina sequencing (Klindworth et al., 2012). Paired-end sequencing was performed with an Illumina MiSeq by the Macrogen company (Macrogen Inc., South Korea) and following the manufacturer's run protocols. Raw sequence data can be accessed from MG-RAST database (accession numbers from mgm4900959.3 to mgm4900970.3).

Samples were processed and analysed by using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline v.1.8.0 software. Raw sequence reads were quality trimmed using the QIIME suite of tools, version 1.8.0 (Caporaso et al., 2010). Low-quality paired-end reads and chimera reads were discarded in operational taxonomic units (OTU) clustering analysis using CD-HIT-OTU. Paired-end reads were assembled using FLASH (Magoč and Salzberg, 2011). Sequences shorter than 120 bp were excluded from further analysis. OTUs were clustered at 97% similarity threshold using UCLUST (v.1.2.22). Taxonomy assignment was performed using GreenGenes (v13.8) as a reference (McDonald et al., 2012). Various alpha diversity indices were estimated based on clusters using Shannon (H′), Simpson (D) and Chao1 and observed species metrics in QIIME software. Clone library coverage based on Good's coverage for an OTU definition (Good, 1953) was determined using 97% identity level.

The R software (R Core Team, 2013) was used to prepare the double hierarchical dendrogram and the heatmap depicting the relative abundance of the top 20 phyla (abundance higher than 1% in at least one sample). The Bray–Curtis dissimilarity matrix was calculated on the full dataset. Average linkage hierarchical clustering was opted for. The heatmap was generated using the "Heatplus" library. Hierarchical clustering was performed using the "vegan" library. The Principal Coordinate Analysis (PCoA) plot was based on Bray–Curtis distance and relative abundances of bacterial and archaeal OTUs, which were used as the dataset.

3. Results and discussion

Nowadays, the Arctic is undergoing massive transformations, including temperature increase, glacier melting, milder winters, less snow and ice cover of land, fiords and Arctic Ocean, and many others. All the above, together with rising tourism and related anthropogenic impact (emissions from ships, planes, growing permanent human settlements and scientific bases) lead to significant, yet still not fully understood changes in the polar environment. It is clear, however, that they result in shifts in the ecosystem: introduction of nutrients (Qu et al., 2017) and other pollutants (Eckert et al., 2018), as well as suspected changes in the microbial community (Wang et al., 2017) and resistome structure (Alexander et al., 2020). In this study, two Arctic lakes were chosen as model objects to reveal differences between "pristine" lake under the natural pressure of a tundra stream and runoff from birds nesting area, versus "anthropogenically influenced" lake being a treated wastewater receiver. Reservoirs near the Polish Polar Station, Hornsund, West Spitsbergen were analysed regarding physicochemical parameters, microbial community composition and antimicrobial resistance of *Enterococcus* spp..

3.1. Physicochemical analysis

Electrical conductivity (EC) generally shows the presence of dissolved salts and in some cases can be used as an indirect indicator of pollution (Ribeiro De Sousa et al., 2014). Biologically productive freshwater typically present EC values of 100–500 μS cm^{-1}, while lower values (<100 μS cm^{-1}) usually suggest oligotrophic (nutrient-poor) conditions (Stewart, 2001). In this study, samples collected from the tested lakes showed EC values from 120 μS cm^{-1} to 211 μS cm^{-1}, with slightly higher EC observed in the sampling points subjected to either anthropogenic (WW-R) or natural, bird-related (L-TS) inflow, up to 211 μS cm^{-1} and up to 155 μS cm^{-1}, respectively (Table 1). Nonetheless, the EC values in lake-related samples fall in the range noted for other aquifers in the area of the Polish Polar Station (Kosek et al., 2019; Nowiński and Wojtasik, 2006) and, as suspected, were significantly lower than those noted for treated wastewater (WW-E, up to 1115 μS cm^{-1}), which is also in the range of typical treated wastewater EC values (see e.g. Prieto et al., 2001).

The temperature of the samples collected from the lakes (L-TS, L-WS and WW-R) was about 7 °C and to some extent, as with other shallow water bodies of this kind, it was linked to the air temperature (Woelders et al., 2018). Mean air temperature during the sampling period (August 2013) was equal to +5.8 °C, which was 1.7 °C higher than the multiannual mean for this month (Polish Polar Station Meteorological Bulletin, 2013). The WW-E temperature was about 18 °C and resulted from the thermal conditions inside the wastewater treatment plant building (set at 20 °C). The pH values ranged from 7.0 to 7.8 in samples collected from Lake 1 and Lake 2, and from 7.2 to 7.5 in WW-E (Table 1). Note that lake acidification was reported as a particular sign of inflow related to the birds' breeding area (González-Bergonzoni et al., 2017; Zwolicki et al., 2013). In this study, the decrease in pH was less profound but was observed at Lake 1 in point L-TS. This site is under the direct influence of the tundra stream, collecting surface runoff from little auk colonies ($pH = 7.1 \pm 0.08$ versus $pH = 7.7 \pm 0.08$ at the L-WS point at the water supply area). In this study, nitrogen and phosphorus in Lake 1 were mostly below the level of detection (Table 1), except ammonia (up to 0.77 mg $N\text{-}NH_4$ L^{-1}) and nitrates (up to 0.40 mg $N\text{-}NO_3$ L^{-1}), which at the L-TS point constituted the main share of total nitrogen (Table 1). This suggests that influence from runoff that is nutrient-rich due to bird droppings was either retained by the surrounding tundra vegetation or diluted by intense rainfalls. In August 2013, during the sampling campaign, exceptionally high rainfall was noted: 179.5 mm per month. It was more than three times the average multiannual (1978–2012) precipitation for August (51.9 mm) and over 50 mm higher than the previous maximum noted in August 2012 (123.8 mm, for more details see the Extended characterisation of the investigated area in supplementary materials).

Increased nutrients concentrations were observed in Lake 2, which serves as a wastewater effluent receiver. The inflow of biogenic substances can highly influence the biochemical potential and microbial

Table 1
Physicochemical parameters of water collected from the Lake 1 (L-TS: tundra stream inflow and L-WS: water supply area) and Lake 2 (WW-R: treated wastewater recipient); the results of wastewater treatment plant effluent (WW-E) were compared with the discharge requirements. Values are given in the format: average±SD.

Parameter	Unit	L-TS	L-WS	WW-R	Requirements for treated wastewater[a]	WW-E
T	°C	7.10 ± 0.60	6.90 ± 0.09	6.30 ± 0.20	–	18.3 ± 0.60
pH	[−]	7.10 ± 0.08	7.70 ± 0.08	7.30 ± 0.14	–	7.30 ± 0.12
EC	μS cm^{-1}	148.5 ± 5.9	129.2 ± 7.6	191 ± 16	–	1074 ± 46
$N\text{-}NH_4$	mg L^{-1}	0.56 ± 0.21	0.12 ± 0.08	1.12 ± 0.40	–	34.2 ± 5.6
$N\text{-}NO_3$	mg L^{-1}	0.29 ± 0.11	< LOD (<0.25)	0.85 ± 0.20	–	6.7 ± 2.1
TN	mg L^{-1}	1.04 ± 0.71	<LOD (<1.0)	2.03 ± 0.55	≤ 30	71.6 ± 9.2
$P\text{-}PO_4$	mg L^{-1}	<LOD (<0.05)	< LOD (<0.05)	0.19 ± 0.09	–	7.4 ± 2.0
TP	mg L^{-1}	<LOD (<0.05)	< LOD (<0.05)	0.25 ± 0.09	≤ 5	8.9 ± 1.9
COD	mg O_2 L^{-1}	< 5	< 5	30.0 ± 5.3	≤ 150	168. ± 21

LOD – limit of detection.
[a] According to Polish Ministry of Maritime Economy and Inland Navigation (2019)

community in such oligotrophic lake, what is discussed further. According to the obtained data, the requirements of treated wastewater discharge were not met (Polish Ministry of Maritime Economy and Inland Navigation, 2019), especially in the case of total nitrogen content (up to 80 mg N L^{-1} in WW-E, Table 1). The efficiency of this wastewater treatment plant before modernisation was investigated in another study (Wilk and Cimochowicz-Rybicka, 2018). The disturbances observed in the wastewater treatment plant operation were connected with the summer season and full occupancy of the Polish Polar Station (up to 45 people in total). As a result, the decrease in hydraulic retention time, weak floc formation and settling, and finally activated sludge biomass washout was observed (for details, see sections 3.2 and 3.3, Supplementary Figs. S2 and S3). As a consequence, a drop of the nitrification/denitrification efficiency was noted. The findings of this study were later used to adapt the wastewater treatment system (done in 2016). However, small scale wastewater treatment plants are generally more prone to failures and problems. They are difficult to operate – partly due to high variability of inflow and load that leads to the lower stability of the system, not only in the polar areas, but even in the mid-latitudes.

3.2. Direct microscopic quantification of the prokaryotic community

In general, a clear relationship was confirmed between the amount of available biogenic compounds and bacterial abundance, cell volume and biomass (Danovaro and Fabiano, 1997; La Ferla et al., 2010, 2014). Therefore, the physical appearance of prokaryotic cells carries (unspecific) information about the trophic status of the aquatic environment. In polar regions, apart from the deficit of nutrients, also low temperature and consecutive periods of very high and very low exposure to solar radiation (polar day and night) are important factors influencing bacterial development (Kirchman et al., 2005; Mueller et al., 2005; Rublee and Bettez, 1995). But even in high Arctic lakes, classified as oligotrophic, bacterioplankton activity is observed, the highest in mid-August (Laybourn-Parry and Marshall, 2003). Thus, microscopic observations play an important role in the evaluation of the activated sludge condition. Incorporated into environmental impact assessment of the wastewater receivers, such analyses could also provide rough information about the microbiological water quality.

In this study, as suspected, all analysed parameters: total prokaryote cell number (TCN), average cell volume (ACV), prokaryote biomass (PB) and LIVE/DEAD ratio (L/D) were lowest in Lake 1 (L-WS, L-TS), followed by the wastewater-related points: the treated wastewater recipient (WW-R) and the wastewater treatment plant effluent (WW-E, Fig. 2, S2 and S3). Importantly, the above parameters obtained for lake-related samples (L-TS, L-WS and WW-R), were higher than in other freshwater samples collected in the Arctic. For instance, the average values of TCN and PB in this study were: 1.16×10^6 cells mL^{-1} and 28.75 μg C L^{-1} in L-TS, 1.21×10^6 cells mL^{-1} and 25.04 μg C L^{-1} in L-WS and 2.31×10^6 cells mL^{-1} with mean biomass 61.24 μg C L^{-1} in WW-R, respectively (Fig. 2). Values reported by Górniak et al. (2016) and Kosek et al. (2018, 2019) in cold proglacial lakes and a brisk glacial river were even one magnitude lower, which reflects the difference with the less turbulent, warmer and more fertile Lake 1 and Lake 2.

Compared with Lake 1, Lake 2 (WW-R) showed higher availability of nutrients (Table 1), probably due to the treated wastewater discharge (WW-E). This can result in an intensification of the primary and bacterial production. Additionally, in both lakes (Lake 1 and Lake 2) the bacterial growth could have been supported by the relatively high temperature noted during the sampling campaign (for details see Supplementary Materials), as several studies underline the influence of temperature on the physical properties of prokaryotic communities (La Ferla et al., 2010; Ntougias et al., 2016). In the case of treated wastewater effluent (WW-E), values of bacterial abundance (up to 5.07×10^6 cells mL^{-1}) and biomass (up to 152.52 μg C L^{-1}) were the highest among tested samples. The LIVE/DEAD assay showed also the highest ratio of live cells in WW-E (15.5% on average), followed by wastewater recipient (WW-R, 10.2%), tundra stream inflow (L-TS, 8.1%) and water supply point (L-WS, 5.9%).

Live and active cells typically constitute up to 80% of the bacterial community in activated sludge biomass (Kocwa-Haluch and Woźniakiewicz, 2011) and are mainly concentrated in sludge flocs. Thus, the elevated abundance of active bacterial cells in the wastewater effluent is usually a sign of biomass washout. In this study, both free-swimming and flocs-related bacteria were observed in WW-E (Supplementary materials, Fig. S2 and S3). It is suspected that, in the studied wastewater treatment plant, small and weak flocs of activated sludge were formed, then easily sheared and subjected to flotation in the final clarifier. This can be principally caused by short hydraulic retention time and insufficient sludge age, causing endogenous metabolism, lack of floc-forming species and/or low production or destruction of extracellular polymers substances. A high concentration of readily degradable substrates and/or the presence of some toxic or inhibitory compounds in wastewater also matters. Those technological problems were confirmed in this study not only by the continuous biomass washout to effluent but also by the deterioration of effluent quality (increase in turbidity, TN, TP and COD values; see Table 1). It is also suggested that the fluorescence microscopy observations of the wastewater treatment plant effluent can serve as a method for identifying treatment efficiency or technological problems, related to, for example, activated sludge washout.

Average cell volume (ACV) is another indicator, which can be linked to the bacterial population activity and dynamics (Cole et al., 1993; Šimek et al., 1994), as well as the nutrients availability. Different bacteria size classes dominate in various environments: small forms prevail in oligotrophic waters, and larger rods in eutrophic (Billen et al., 1990). Small cells (around 0.12 μm^3) are considered to be the most active (Gasol et al., 1995). Also the limited residence time of bacteria in the ecosystem influences their development (Lew et al., 2016) – in this study prokaryotic ACV around 0.14 μm^3

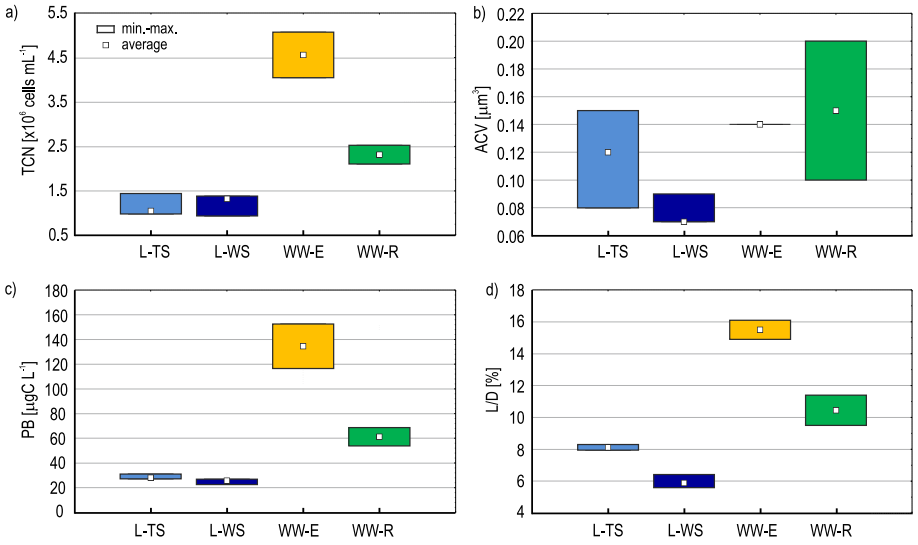
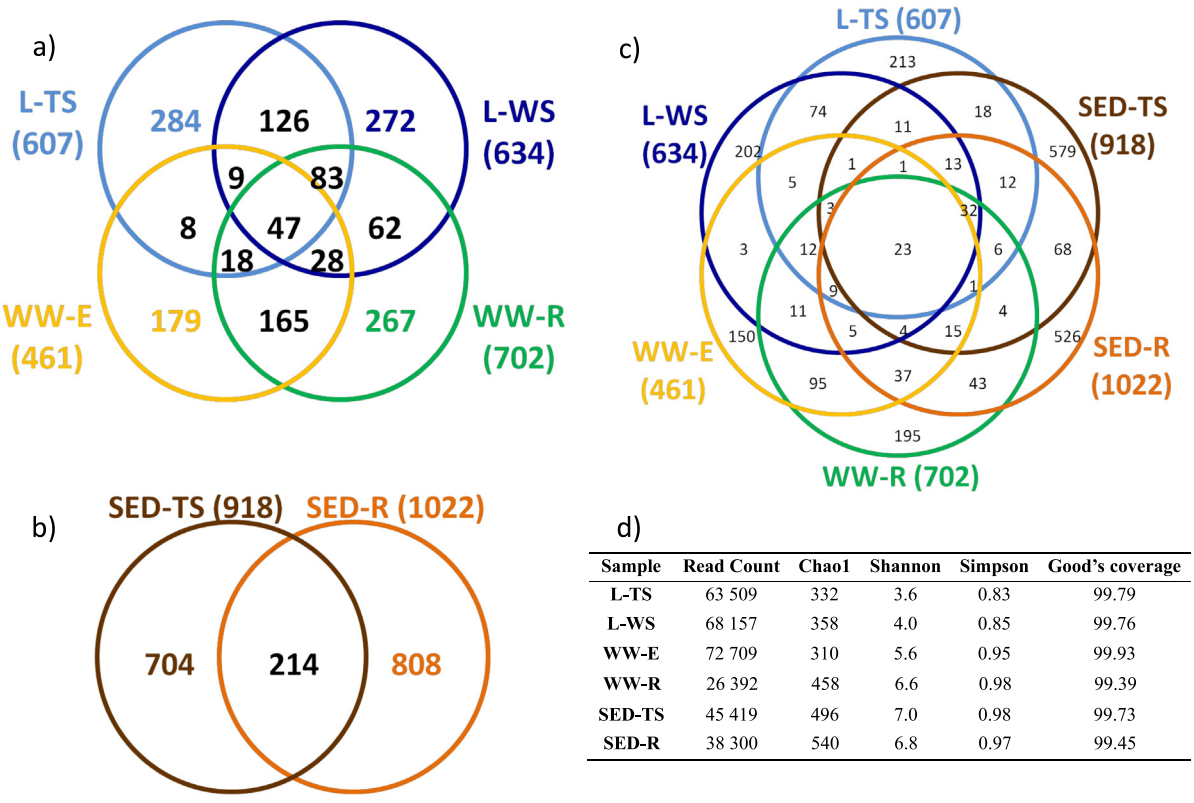


Fig. 2. Microscopic analysis results in water and wastewater samples: a) total prokaryotic cell number (TCN), b) average cell volume (ACV), c) prokaryotic biomass (PB) and d) prokaryotic activity – live cells expressed as a percentage of the total community (L/D).

was noted in WW-E, which reflects both intensive bacterial development in a nutrient-rich environment and the impact of continuous flow conditions. The largest ACV range, which is observed in L-TS and WW-R samples, seems to result from nutrient supply of natural (L-TS) or anthropogenic (WW-R) origin and more stagnant conditions than in the wastewater treatment system, which favour growth of microorganisms. Cell volume variability (Fig. 2b), which is especially noted in WW-R and L-TS, could also reflect the presence of two kinds of prokaryotic cells in the Arctic lake: autochtonous and discharge-related allochthonous bacteria.

3.3. Microbial community composition and diversity indices

The Shannon and Simpson diversity indices are a proxy for richness and evenness. They were found to be the lowest for L-TS and L-WS samples (3.6–4.0 and 0.83–0.85, respectively), intermediate for WW-E (5.6 and 0.95) and highest for WW-R and both sediment samples (SED-TS and SED-R; 6.6–7.0 and 0.97–0.98, Fig. 3d). The Chao1 richness estimator predicts the total number of OTUs, but it also takes into account the numbers of singletons and doubletons (species represented by exactly one or two individuals, respectively), therefore it is highly influenced



d)

Sample	Read Count	Chao1	Shannon	Simpson	Good's coverage
L-TS	63 509	332	3.6	0.83	99.79
L-WS	68 157	358	4.0	0.85	99.76
WW-E	72 709	310	5.6	0.95	99.93
WW-R	26 392	458	6.6	0.98	99.39
SED-TS	45 419	496	7.0	0.98	99.73
SED-R	38 300	540	6.8	0.97	99.45

Fig. 3. Venn diagrams displaying the number of OTUs shared between the samples: a) water and wastewater samples only, b) sediment samples only, c) all samples. Numbers in brackets refer to the total number of identified OTUs in the sample. Diversity and richness estimators for Illumina libraries are shown in Fig. 3d.

by rare OTUs and presents a slightly different pattern. Chao1 was the lowest for WW-E (310) and the highest for sediment samples: SED-TS and SED-R (496 and 540, respectively, Fig. 3d). In each sample the Good's coverage indicates that the range of bacterial diversity is well represented (over 99%).

A total of 2760 OTUs were identified from 314,486 sequences (average length of 428 bp), which were achieved in the present study for 6 analysed samples. For water and wastewater samples, 47 OTUs were common (Fig. 3a) and sediment samples shared 214 OTUs (Fig. 3b). Among all the OTUs, 23 were present in all the samples (Fig. 3c) and they belonged to *Actinobacteria*, *Bacteroidetes*, *Parcubacteria*/OD1, *Proteobacteria*, as well as *Saccharibacteria*/TM7 and *Verrucomicrobia*, which are present in the samples in lower relative abundance. The highest amount of unique OTUs was observed in sediments: 579 out of 918 OTUs in SED-TS and 526 out of 1022 OTUs in SED-R (Fig. 3c). One hundred and fifty OTUs were unique to the wastewater sample and represented mainly the phyla *Dojkabacteria*/WS6 and *Parcubacteria*/OD1, as well as *Chloroflexi*, *Firmicutes*, *Proteobacteria* and *Microgenomates*/OP11 in smaller shares (Fig. 4 and Fig. 5).

Taxonomy-based analysis indicated that *Bacteria* constituted a majority, and *Archaea* less than 0.02% of the total microbial community in each sample, except for sediments collected from tundra stream inflow

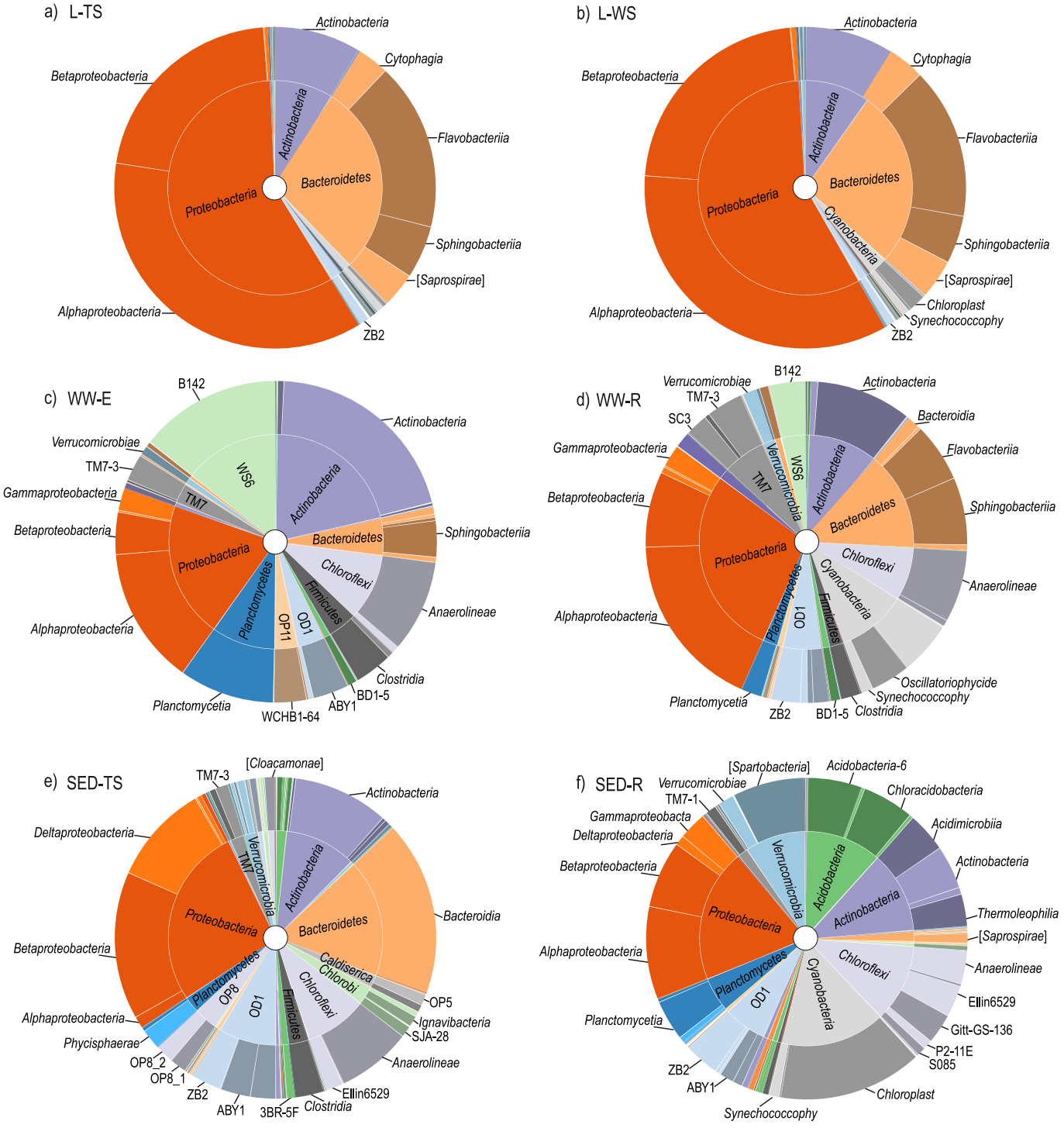


Fig. 4. Bacterial community composition of the samples on phylum (inner ring) and class level (outer ring).

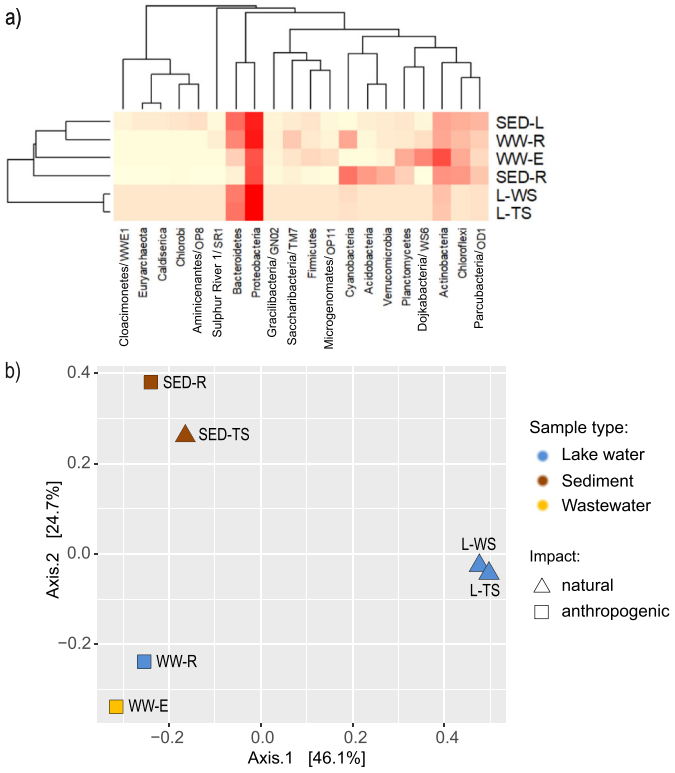


Fig. 5. a) Heatmap of microbial community richness at the phylum level. Colour code indicates relative abundance, ranging from yellow (low) to red (high). b) Principal Coordinates Analysis for microbial community OTUs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(SED-TS), where *Archaea* accounted for 2.54%. In the case of *Bacteria*, their community consisted of 55 phyla, 37 of which were abundant only in minor shares of less than 1% in each sample. Unassigned sequences (not assigned to any Kingdom) represented fewer than 0.6% and were most abundant in sediment samples, which is in agreement with the literature that indicates under-representation of the soil taxonomy in the databases (Bulgarelli et al., 2012; Gans et al., 2005).

In the case of wastewater effluent (WW-E), 10 core phyla constituted over 96% of the community. The most abundant were *Actinobacteria* and *Proteobacteria* (21% each), followed by *Dojkabacteria*/WS6 (14%), *Chloroflexi* and *Planctomycetes* (10% each), with smaller shares of *Bacteroidetes*, *Firmicutes*, *Parcubacteria*/OD1, *Microgenomates*/OP11 and *Saccharibacteria*/TM7 (3–6% each, Fig. 4c). Some of those phyla and their representatives were also detected in major shares in wastewater treatment plant bioreactors (Saunders et al., 2016), including those serving municipalities in the Arctic Circle (eg. *Bacteroidetes*, *Firmicutes* and *Rhizobiales* from *Alphaproteobacteria*, as well as *Comamonadaceae* from *Betaproteobacteria*) (Gonzalez-Martinez et al., 2018). Others (e.g. *Microgenomates*/OP11, *Parcubacteria*/OD1 and *Saccharibacteria*/TM7) were also found in various environments other than in activated sludge systems, under anoxic (nitrate and sulphate reducing) and anaerobic conditions (Elshahed et al., 2007; Gihring et al., 2011; Harris et al., 2004; Peura et al., 2012).

The recipient (WW-R) to some extent mirrors the core phyla from WW-E, but in different shares (Fig. 4d). This suggests that besides affecting the chemical characteristic (see section 3.1), the wastewater discharge also influenced the microbiology of Lake 2. In WW-R, *Proteobacteria* (29%) and *Bacteroidetes* (15%) were followed by *Actinobacteria* and *Cyanobacteria* (10.5% each), *Chloroflexi* (8%), *Saccharibacteria*/TM7 and *Parcubacteria*/OD1 (6–7%), with smaller shares of *Dojkabacteria*/WS6, *Planctomycetes*, *Firmicutes* and *Verrucomicrobia* (2–4%). The influence of treated wastewater (WW-E) on the recipient (WW-R) can be seen not only at the phylum level, but also at lower taxonomic levels (258 shared OTUs, among which 165 were unique to WW-E and WW-R, Fig. 3a). Particularly high abundances (2–5%) in both samples were noted for orders from *Alpha*-subdivision (*Proteobacteria* phylum): *Rhizobiales* as well as *Caulobacterales* with the activated-sludge-related genus *Phenylobacterium*. *Isosphaeraceae* and *Pirellulaceae* families (*Planctomycetes* phylum) were most abundant in WW-E, WW-R and SED-R (0.7–5.5%). They are usually related to multistage activated sludge process and found mainly in aeration basins (Zheng et al., 2016), so their presence indirectly confirms their possible washout from the wastewater treatment plant with activated sludge flocs (See supplementary materials, Fig. S2 and S3). The *Nocardioidaceae* family from the *Actinobacteria* phylum were most abundant in WW-E (12.5%) and WW-R (3.4%). Their representatives are widespread in natural and polluted environments and are known for their ability to decompose a wide range of organic matter (including at low temperatures). Therefore, they are suspected of playing a significant role in degradation processes (Tóth and Borsodi, 2014). However, in this study, mostly unclassified genera of *Nocardioidaceae* family have been noted. Non-phototrophic *Caldilineaceae* and *Anaerolinaceae* families of *Chloroflexi* phylum, related to municipal and domestic wastewater treatment systems (Saunders et al., 2016; Zhang et al., 2017) were abundant (6–7% and 1%, respectively) in wastewater related samples (WW-E and WW-R), while in Lake 1 they did not exceed 0.1%. A similar tendency was observed for gut-related *Clostridia* (phylum *Firmicutes*) and potentially human-associated clade TM7–3 of the *Saccharibacteria*/TM7 phylum. The B142 class from the *Dojkabacteria*/WS6 phylum constituted over 14% of WW-E and 3.6% of WW-R, but was present only in minor shares (<0.3%) in Lake 1 (L-TS and L-WS samples). The order *Sphingobacteriales* (phylum *Bacteroidetes*) was present in similar shares in Lake 1 and Lake 2, as well as in treated wastewater (~5%), though wastewater-related samples (WW-E and WW-R) contained mostly unknown taxa, whereas Lake 1 was dominated by the *Sphingobacteriaceae* family, including unknown species from the *Pedobacter* genus, which are common in various habitats, from soil and freshwater to alpine glaciers (Gordon et al., 2009; Margesin et al., 2003; Roh et al., 2008; Shivaji et al., 2005).

In Lake 2, the aforementioned influence of WW-E on WW-R was visible also in terms of its more diversified microbial community than Lake 1, which was indicated by biodiversity indices (Fig. 4d). In the case of Lake 1, points L-TS and L-WS were dominated by only three phyla: *Proteobacteria* (57–58%) *Bacteroidetes* (27%) and *Actinobacteria* (9–10%), altogether constituting over 93% of the community (Fig. 4a and 4b). It was, however, suspected that microbial community, at least at point L-TS, would mirror to some extent the impact of tundra stream inflow and the nearby bird breeding area (mainly of a little auk colony). Nevertheless, chemical data were similar for both water samples from Lake 1. It was also confirmed by the taxonomic data showing that L-WS and L-TS core microbiota were characterised by similar microbial composition up to genus level, with minor differences noted for the *Cyanobacteria* phylum. This indicated that the bird-droppings-related runoff was retained by the tundra vegetation surrounding Lake 1 (mainly lichens and mosses) or diluted by intense rainfalls (see section 3.1).

Interestingly, the core phyla of both freshwaters (Lake 1 and Lake 2) were mostly represented by *Alpha*- and *Beta*- subdivisions of *Proteobacteria*; *Flavobacteria* and *Sphingobacteria* belonging to *Bacteroidetes*, as well as *Actinobacteria* classes (Fig. 4a, 4b, 4d, Table S1). These taxa dominate in freshwater (Michaud et al., 2012; Rozmarynowycz et al., 2019), as well as in Arctic river–lake systems located around the Polish Polar Station (Kosek et al., 2019; Ntougias et al., 2016). The prevalence of *Actinobacteria*, *Alpha*- and *Betaproteobacteria* with a high relative abundance of *Burkholderiales* and *Sphingomonadales* was also found in an endophyte population in the Arctic tundra

(Nissinen et al., 2012). *Acidobacteria* have frequently been reported as predominant taxa in Canadian, Alaskan and Siberian Arctic soils (Campbell et al., 2010; Neufeld and Mohn, 2005; Rawat et al., 2012; Wallenstein et al., 2009) and are regarded as an indicator of tundra influence (Männistö et al., 2013), but in this study, they did not exceed 0.5% in Lake 1 and Lake 2. The Lake 2 (WW-R) microbial community, however, contained significant shares of endophytic classes *Oscillatoriophycideae* (3.9%, *Phormidium* genus), *Synechococcophycideae* (1.2%, mostly genus *Leptolyngbya*) and other unclassified *Cyanobacteria* (5.4%), which were less abundant in Lake 1 (Table S1). The presence of these bryophyte and plant-related taxa, as well as *Pseudanabena* species, was also noted by Richter et al. (2018) in the fertile, ornithogenic and moss-dominated area around the Polish Polar Station. Undoubtedly, in Lake 2 (WW-R point) cyanobacteria growth could be supported by the release of nutrients with wastewater treatment plant effluent (WW-E), which was confirmed by the presence of the aforementioned nitrophilous taxa.

Note that, despite continuous ammonia discharge with the WW-E (up to 40 mg $N\text{-}NH_4$ L^{-1}), it was not accumulated in Lake 2 (<1.2 mg $N\text{-}NH_4$ L^{-1} in point WW-R). This can be related to the dilution factor as well as microbial activity. The ammonia- and nitrite-oxidising microorganisms were present in very low shares in both lakes, and did not exceed 0.1% in WW-R and 0.01% in L-TS and L-WS. However, even in ammonia-rich niches such as wastewater, the relative abundance of the ammonia/nitrate-oxidising community is low (Saunders et al., 2016). According to the obtained results the main role in the oxidation of ammonia to nitrite in WW-R was played by *Nitrosomonas* spp., with *Nitrospira* as possible nitrite-oxidising bacteria (NOB). However, a metabolic function of *Nitrospira* in the environment is difficult to assign, since *Nitrospira* members could perform full nitrification, nitrite oxidation, or other alternative pathways beyond the nitrogen cycle (Koch et al., 2015). Anaerobic ammonium oxidation (anammox) bacteria *Candidatus Brocadia* were detected only in Lake 1, which reflects its possible origin from occasionally deoxygenated tundra soil and decomposing plants transported by surface runoff (Kosek et al., 2019). The absence of *Nitrobacter*, noted in our study, was reported in the Arctic freshwater system also by Ntougias et al. (2016), but the significant shares of unknown genera of the *Bradyrhizobiaceae* family (up to 1% in WW-E) and the *Rhizobiales* order (up to 3.6% in WW-E, Table S1) suggests that NOB were very likely represented in the samples and their low detection could mainly be ascribed to the limited robustness of gene-fragment assignment to lower taxonomic levels.

Besides anammox, denitrification is another process releasing nitrogen to the atmosphere. A wide variety of heterotrophic facultative anaerobes are capable of oxidising organic compounds via nitrate respiration. Thus, in this study possible denitrifiers may belong to genera such as *Flavobacterium* (2.4–2.8% in WW-R, L-TS and L-WS) and/or *Clostridium* (0.6–1.5% in WW-R and sediment samples, Table S1), and also to representatives of the *Actinomycetales* family (phylum *Actinobacteria*), the *Bacillus* genus (phylum *Firmicutes*) or the *Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria* class of the *Proteobacteria* phylum.

In the studied lakes, apart from biogenic compounds, non-indigenous microorganisms (e.g. human- and animal-related bacteria) too can be introduced. Among faecal indicators, bacteria from *Escherichia* genus were noted in each sample, with the highest relative abundance in WW-E (0.1%) followed by WW-R (0.06%), while in Lake 1 samples (L-TS and L-WS) they did not exceed 0.01%. A similar tendency was noted for other faecal indicators – members of *Enterococcus* spp. (WW-E – 0.09%, WW-R – 0.04% and < 0.01% in L-TS and L-WS). Additionally, one of the most abundant commensal bacteria in the human gut microbiota, constituting even 5% of the intestine community in a healthy adult (Miquel et al., 2013), *Faecalibacterium prausnitzii*, was also found, but only in WW-E and in a minor share (<0.01%). Its absence in WW-R can be due to the fact that long survival of *F. prausnitzii* outside the human gut is very unlikely, mainly due to sensitivity to oxygen (El Hage et al., 2017). Cellulose-degrading *Ruminococcus*, possibly associated both with human- and reindeer-gut microbiota, was found in similar abundances in both lakes (up to 0.01%) and treated wastewater (0.04%). Bacterial sequences potentially associated with bird faeces contained species identified as responsible for fish infections (*Acinetobacter johnsonii* or *Vagococcus salmoninarum*), indicating the possible guano impact of some piscivorous bird species other than the planktivorous little auk.

In this study, sediments from a tundra stream (Lake 1, SED-TS) and wastewater discharge (Lake 2, SED-R) were also collected. According to the obtained data, the microbial communities of SED-TS and SED-R differed from each other and the other samples (treated wastewater [WW-E] and lake waters [WW-R, L-TS and L-WS]). This was indicated by the largest share of unique OTUs in SED-TS and SED-R (Fig. 3c) and the highest value of diversity indices (Shannon, Simpson and Chao1, Fig. 3d). The bacterial communities in both sediment samples were composed mainly of *Proteobacteria* (20–26%), *Actinobacteria* (11–12%), *Parcubacteria*/OD1 (7–8%) and *Chloroflexi* (9–12%, Fig. 4e, f). However, in SED-TS, *Bacteroidetes* represented 17% of the community, while in SED-R they were replaced by other phyla: *Cyanobacteria* (16%), *Verrucomicrobia* (9%) and *Acidobacteria* (11%). Soil- and tundra-related *Acidobacteria* were more abundant in the wastewater-discharge-related sediments (SED-R, 11%) than in the tundra stream inflow (SED-TS, 1.5%). The development of *Cyanobacteria* (14%) in SED-R, can be favoured by the supply of nutrients by the treated wastewater. In the SED-TS sample, where *Cyanobacteria* were rare (0.12%), anaerobic sediment-related archaeal methanogens were noted (genus *Methanosaeta*, 1.4%, and *Methanoregula* – 0.49%, Table S1). Similarly, sulphate-reducing *Deltaproteobacteria* were particularly abundant at the tundra stream inflow (9.7% in SED-TS vs 1% in SED-R), consisting mainly of *Desulfobacterales* (*Desulfobulbaceae* family), *Desulfuromonadales* (*Geobacteraceae* family members, including the iron-reducing *Geobacter* genus) and *Syntropobacterales* (*Desulfobacca* and *Desulfomonile* genera).

The hierarchical heatmap at the bacterial phylum level reveals the dominance of *Proteobacteria* among all the samples, as well as the site-specific presence of *Actinobacteria* and *Dojkabacteria*/WS6 phyla in WW-E, and *Cyanobacteria* in SED-R. Two clusters confirm the closest resemblance between Lake 1 water samples (L-TS and L-WS), these being different from sediment and wastewater-related samples (Fig. 5a), which is also shown by PCoA analysis (Fig. 5b). Sediment samples differ from the water and wastewater samples, though neither is closely related to the other.

3.4. Prevalence and identification of Enterococcus spp.

The transmission of human and animal-related bacteria and their genetic elements is possible mainly by faecal contamination of the environment, and thus in this study the presence of faecal indicator *Enterococcus* spp. was tested in wastewater treatment plant effluent (WW-E) and in lake-related samples (WW-R, L-TS and L-WS). As suspected, among the studied points *Enterococcus* spp. were the most abundant in WW-E – from 0.7×10^3 CFU/100 mL to 1.9×10^3 CFU/100 mL. This is, however, rather low compared to other wastewater treatment plants' effluents (Sadowy and Luczkiewicz, 2014). In the treated wastewater receiver (WW-R) enterococci varied from 11 to 150 CFU/100 mL and their abundance was, in general, higher than in Lake 1: up to 30 CFU/100 mL in L-TS, and occasionally noted in L-WS, (<1 CFU/100 mL). Note that the presence of *Enterococcus* spp. was confirmed not only by the culture-dependent approach, but also by metagenomics (minor shares, less than 0.1%, see section 3.3). Nonetheless, compared to the New Bathing Directive (2006/7/EC) requirements, both tested lakes (points L-TS, L-WS, WW-R) represented excellent water quality in terms of enterococcal presence (below 200 CFU/100 mL).

Among cultivated enterococcal strains, 76 were isolated from the samples (17 from L-TS, 23 from L-WS, 16 from WW-E and 20 from

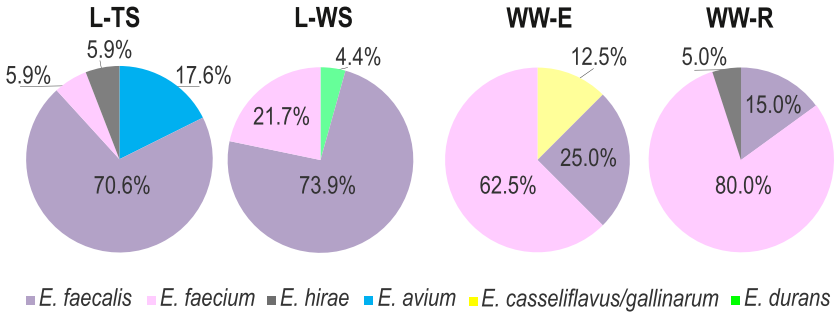


Fig. 6. Identification of *Enterococcus* spp. isolated from wastewater effluent (WW-E) and two lakes: under natural (L-TS, L-WS) and anthropogenic (WW-R) impact.

WW-R), then biochemically identified (Fig. 6) and tested for antimicrobial susceptibility (Fig. 7). Of 76 isolates, 36 were identified as *E. faecalis* (47.4%), 32 as *E. faecium* (42.1%) and the remaining as *E. avium* ($n = 3$; 3.9%), *E. hirae* ($n = 2$; 2.6%), *E. durans* ($n = 1$; 1.3%) and *E. casseliflavus/gallinarum* (n = 2; 2.6%). According to the obtained results, two species, *E. faecalis* and *E. faecium*, comprised 76–95.6% of all enterococcal isolates in a single sample, as they belong to the autochthonous microbiota of human and animal gastrointestinal tracts (Lebreton et al., 2014; Wu et al., 2019). Interestingly, *E. avium*, commonly related to birds' intestinal tract (Yu et al., 2019), was observed mainly in Lake 1 at the tundra stream discharge (L-TS). *E. faecium* was predominant in WW-E (62.5%) and WW-R (80%), while *E. faecalis* dominated in the L-TS (70.5%) and L-WS (73.9%) samples. The reason of such dominance is not fully clear and can be related to the limited number of isolates. However in general, this is in agreement with Zaheer et al. (2020), who suggested that to some extent enterococci show niche specificity, and for this reason they can be used as indicator bacteria in antimicrobial resistance studies.

3.5. Antimicrobial resistance of Enterococcus spp.

The dissemination of antimicrobial resistance in polar regions requires attention, due to the observed rapid increase in human activity in this area, and other environmental changes. Wild birds that migrate annually to the Arctic for breeding are also increasingly studied as vectors for the transmission of resistant bacteria and resistance genes (Hernandez et al., 2010; Radimersky et al., 2010).

In this study, the susceptibility of *Enterococcus* spp. isolates was assessed against 10 antimicrobial agents and categorised according to the clinical breakpoints and epidemiological cut-off values (ECOFFs) provided by EUCAST (EUCAST, 2020, Fig. 7). The main purpose of clinical breakpoints is to predict clinical efficacy of antimicrobial therapy, while the ECOFF is defined as MIC differentiating the wild-type bacteria from those that have an acquired form of resistance. The clinical resistance among tested enterococci was noted for Nitrofurantoin (MIC >64 mg L^{-1}); note that clinical breakpoints for nitrofurantoin are valid only for *E. faecalis*. Nitrofurantoin is a bactericidal antimicrobial agent used in uncomplicated urinary tract infections (Schmiemann et al., 2012). Clinical breakpoints obtained in this study indicated that resistance to nitrofurantoin was detected only in the wastewater treatment plant effluent (WW-E) in 14.3% of *E. faecalis* isolates. However, MIC distribution evaluated for nitrofuranoin (Fig. 7) showed that *E. faecalis* isolates with MIC >32 mg L^{-1} (above ECOFF value) constituted 59.2% of isolates in WW-E and 33.3% in WW-R (treated wastewater recipient), which is followed by tundra stream discharge (8.3% in L-TS). None of the *E. faecalis* isolates with MIC above the ECOFF value were noted in the area of the water supply system (L-WS).

In the case of *E. faecium*, clinical isolates have already been reported to rarely be resistant to nitrofurantoin (Toner et al., 2016), as also confirmed in this study (Fig. 7), since no isolate with acquired resistance (MIC >256 mg L^{-1}) to nitrofurantoin was detected. Interestingly, resistance to nitrofurantoin can be mediated via plasmids and chromosomal mutations, and resistance among clinically isolated *Enterococcus* spp. has increased in recent years from near zero to 40% (Toner et al., 2016). Additionally, both resistance genes and mobile genetic elements have shown similarity in animals and humans, so transmission of resistance through zoonotic pathogens and commensal food-borne bacteria is possible.

In this study, isolates with MIC above the ECOFF value were also noted for moxifloxacin (MIC >1 mg L^{-1}, *E. facealis* in L-TS: 8.3% and WW-E: 14.3%) and erythromycin (MIC >4 mg L^{-1}) among *E. faecalis* (14.3%) and *E. faecium* (22.2%) in WW-E. Note that the remarkable capacity of *Enterococcus* spp. to acquire resistance to macrolides caused that antimicrobial agents from this chemical class (including erythromycin) are no longer used to treat enterococcal infections (lack of clinical breakpoints, Fig. 7), but they are still in use to treat other emerging infections (EUCAST, 2020). Additionally, data of this study shown that regardless of sampling point, isolates of *E. faecalis* tend to be more susceptible than *E. faecium* to tested beta-lactam agent – ampicillin – similarly as reported among clinical isolates, where most *E. faecium* isolates are ampicilin-resistant (MIC ≥8 mg L^{-1}).

Due to the limited number of isolates tested in this study, no general conclusion can be drawn. But bacteria related to humans and wildlife (including migratory birds) should be monitored to better elucidate both their survival and possible dissemination of antimicrobial resistance. This is of special importance in polar areas, where bacterial fitness cost connected with the collection of resistance determinants could be justified by the presence of other environmental stressors (e.g. UV light presence/absence). All the above are also important in terms of climate change and increasing anthropogenic impact in polar regions.

4. Conclusions

Nutrient transport and cycling in polar lakes are highly influenced by catchment area. In this study, the microbial communities at the tundra stream discharge and the water supply point in Lake 1 were confirmed to bear the closest resemblance and suggested that the nutrient-rich runoff from birds nesting area was retained by the surrounding tundra vegetation or diluted by intense rainfalls. In the case of Lake 2, the effluent from the wastewater treatment plant directly increased the diversity of the microbial community, both by introducing wastewater-related bacteria and by supplying the receiver in nutrients, which may play a significant role in typically oligotrophic Arctic lakes. Also, as most microbiological processes are temperature-related, we can expect that climate changes can accelerate biochemical cycles in Arctic lakes being amended by nutrient inflow. The microscopic observations also confirmed an increase in all tested parameters in Lake 2, such as: total prokaryotic cell number, average cell volume, prokaryotic biomass and live cell percentage. The presence of *Enterococcus* spp. and their antibiotic resistance highlights the importance of wastewater treatment processes in the dissemination of human-associated microbiome and resistome. In polar areas in particular, which are increasingly being visited and inhabited by people, the introduction of wastewater-related, non-indigenous microorganisms justifies the need for advanced

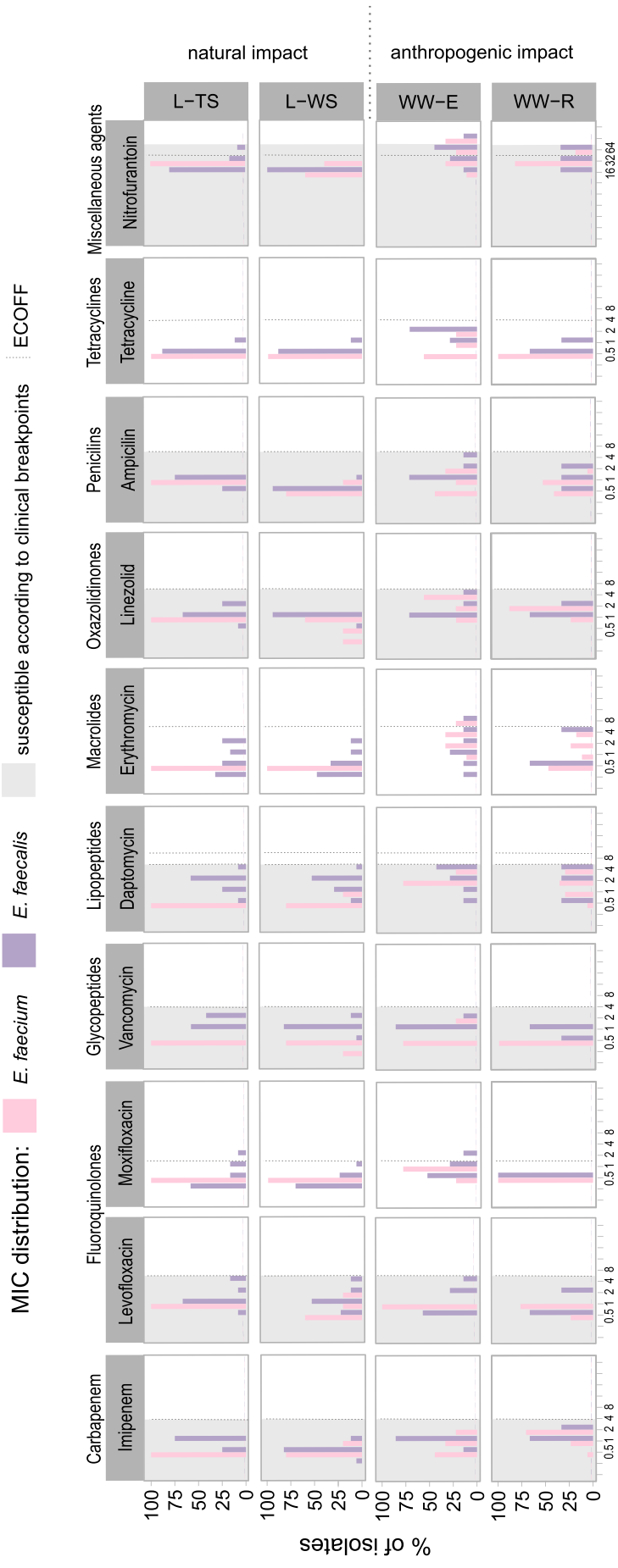


Fig. 7. Distribution of Minimal Inhibitory Concentration (MIC), in milligrams per litre, for the studied *E. faecium* and *E. faecealis*. Clinically susceptible strains are shown on grey field. ECOFFs (epidemiologic cutoff values) for both species are marked as dotted lines. For daptomycin, different ECOFF values are set for *E. faecalis* (4 mg L^{-1}) and *E. faecium* (8 mg L^{-1}). For nitrofurnatoin, clinical breakpoint and ECOFF that are shown on the graph are valid only for *E. faecalis*; ECOFF for *E. faecium* is 256 mg L^{-1}, while clinical breakpoints are not defined.

treatment methods in treatment processes. Analysis of microbial community structure combined with bacterial antibiotic resistance analysis (in wastewater as well as water and sediments of the recipient), provide an insight into the short- and long-term changes posed on the aquatic ecosystems by the wastewater discharge. Detailed monitoring should help to identify and understand how anthropogenic and natural factors impact the functioning of polar niches. Defining so-called 'baseline conditions' is crucial in implementing the necessary regulations related to local human activity.

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CRediT authorship contribution statement

Agnieszka Kalinowska: Methodology, Software, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Katarzyna Jankowska:** Methodology, Software, Investigation, Resources, Writing - review & editing, Visualization, Supervision, Funding acquisition. **Sylwia Fudala-Ksiazek:** Conceptualization, Validation, Investigation, Resources, Supervision, Funding acquisition. **Mattia Pierpaoli:** Software, Formal analysis, Data curation, Visualization. **Aneta Luczkiewicz:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Manuscript III

Kalinowska A., Pierpaoli M., Jankowska K., Fudala-Książek S., Remiszewska-Skwarek A., & Łuczkiewicz A. (2022)

Insights into the microbial community of treated wastewater, its year-round variability and impact on the receiver, using cultivation, microscopy and amplicon-based methods.

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Insights into the microbial community of treated wastewater, its year-round variability and impact on the receiver, using cultivation, microscopy and amplicon-based methods

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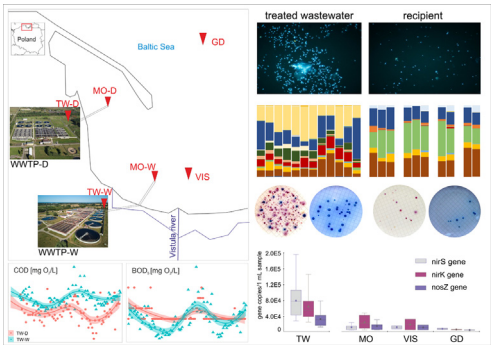
HIGHLIGHTS

- WWTP effluent was studied together with its marine recipient and reference points.
- Microbial quality deterioration of the WWTP effluent was observed in winter.
- Microbial community of the effluent was shaped by the optimization of WWTP processes.
- Activated sludge community was discharged with the treated wastewater to the recipient.
- Nitrogen-cycling genes were disseminated in environment with treated wastewater.

GRAPHICAL ABSTRACT



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Nutrient discharge

ABSTRACT

Apart from chemical constituents, wastewater treatment plant (WWTP) effluents also release microorganisms that can be important to the receiving water bodies either from a sanitary point of view, or taking to the account the biogeochemical potential of the recipients. However, little is known about the treated wastewater microbial community, its composition, seasonal changes, functions and fate in the waters of the receiver. Thus, this study presents a synergistic approach coupling new and traditional methods: analytical chemistry, classical microbiology (cultivation- and microscopy-based methods), as well as Next Generation Sequencing and a quantitative real-time polymerase chain reaction (qPCR). The results show that in terms of bacterial community composition, treated wastewater differed from the environmental samples, irrespectively if they were related or unrelated to the WWTP effluent discharge. The canonical correspondence analysis (CCA) taking into account chemical parameters and taxonomical biodiversity indirectly confirmed the seasonal deterioration of the treated wastewater quality as a result of temperature-driven change of activated sludge community structure and biomass washout (observed also by DAPI staining). Despite seasonal fluctuations of total suspended solids and inter-related parameters (such as COD, BOD, TN, TP), the treated wastewater quality remained within current discharge limits. It was due to treatment processes intensively adjusted

Abbreviations: WWTP-W, Wastewater Treatment Plant Gdansk-Wschod; WWTP-D, Wastewater Treatment Plant Gdynia-Debogorze; TW, treated wastewater; TW-W, treated wastewater from WWTP-W; TW-D, treated wastewater from WWTP-D; MO, marine outfall; MO-W, marine outfall of WWTP-W; MO-D, marine outfall of WWTP-D; VIS, Vistula River estuary; GD, Gdansk Deep; BOD, biochemical oxygen demand; COD, chemical oxygen demand; TSS, total suspended solids; TN, total nitrogen; TP, total phosphorus; DEFT, Direct Epifluorescent Filter Technique; TCN, total (prokaryote) cell number; PB, prokaryote biomass; ACV, average cell volume; OTU, operational taxonomic unit; NGS, Next Generation Sequencing; CCA, canonical correspondence analysis.

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by WWTP operators, particularly those necessary to maintain an appropriate rate of autotrophic processes of nitrification and to support biological phosphorus removal. This can explain the observed microbiome composition similarity among WWTP effluents at high taxonomic levels. Obtained data also suggest that besides wastewater treatment efficiency, WWTP effluents are still sources of both human-related microorganisms as well as bacteria equipped in genes involved in N-cycling. Their potential of participation in nutrients cycling in the receivers is widely unknown and require critical attention and better understanding.

1. Introduction

In urban areas, wastewater treatment plants (WWTPs) usually receive wastewater from households, offices, hospitals, and local industries. Regardless of the type of sewage network, it is clear that WWTPs are crucial in protecting the water resources and other ecosystems from chemical contaminants as well as human-related fecal material (Crini and Lichtfouse, 2019). Thus, adopted in 1991 the Urban Waste Water Treatment Directive (UWWTD, 91/271/EEC) has already aimed to protect the environment from untreated or inadequately treated wastewater, settling the standards for collection and discharge. In general, the Member States have been required to treat the wastewater in agglomerations of ≥2000 population equivalents (PE) to reduce, suspended solids, organic matter (measured as biochemical and chemical oxygen demand; BOD and COD, respectively) and nutrients (nitrogen and phosphorus compounds).

To fulfill the current requirements the wastewater is usually treated by combining mechanical and biological processes. The latter ones are mainly based on activated sludge technology, which employs mixed microbial consortium, enables degradation of the organic pollutants, and is involved in nutrient cycling. Simultaneously wastewater treatment processes reduce the fecal bacteria load with effectiveness reaching usually >90% (Garcia and Bécares, 1997; Reinoso et al., 2008). But even if the removal rate reaches 99.99%, the bacteria of human intestines' origin are not completely removed, since their initial load, expressed by fecal indicators, varied in the general range of 10^6–10^9 per 100 mL (George et al., 2002; Lucena et al., 2004; Łuczkiewicz et al., 2010). For this reason, it is reported that conventional treatment systems are still the source of pathogens (Dias et al., 2019; Ju et al., 2016; Lucena et al., 2004; Ottoson et al., 2006) and other bacteria of concerns, also those carrying resistance genes (Łuczkiewicz et al., 2010; Rizzo et al., 2013; Sadowy and Luczkiewicz, 2014; Tennstedt et al., 2003; von Wintersdorff et al., 2016). Even so, the sanitary quality of treated wastewater is obligatorily analyzed only when reused in agriculture (Dias et al., 2019). Thus, disinfection of wastewater frequently is not required.

Until now, the attention of WWTP operators and scientists has been focused mainly on wastewater treatment processes efficiency, which allows keeping the chemical discharge requirements. Therefore the community composition and biochemical potential of the activated sludge in bioreactors has been investigated more frequently (Albertsen et al., 2012; Saunders et al., 2016; Wagner et al., 2002) than the WWTP outflow (Mansfeldt et al., 2020), which is still largely unexplored area. It is also important to note that in temperate climate zones, cold winter months are highly challenging for activated sludge processes. Especially seasonal decrease of nitrification rate, which is performed by autotrophic bacteria, is observed. The disruptions of wastewater processes may, among others, also hinder the activated sludge settling and its separation from the final effluent in the secondary clarifier, e.g.: due to the presence of filamentous bulking or small, easily sheared pin-flocks (Guo et al., 2016; Morgan-Sagastume et al., 2008). As a consequence, the flocks enter the WWTP effluents and deteriorate their chemical and microbial quality. The composition of dispersed biomass (Do et al., 2019) and the fate of functional genes, carried by those bacteria in the receiver, are almost unknown.

Additionally, it has been already proofed, that the composition of bacterial communities in WWTP effluent can potentially alter the receiving ecosystem (Atashgahi et al., 2015). Indeed in reservoirs the WWTP effluents resulted in both an increase (García-Armisen et al., 2014; Kalinowska et al., 2020; Price et al., 2018; Wakelin et al., 2008) and a decrease (Drury et al., 2013; Lu and Lu, 2014) of bacterial communities diversity. Evaluation of the Urban Waste Water Treatment Directive, prepared by the European Commission, indicated that load of the targeted pollutants discharged via treated wastewater from urban point sources decreased significantly between 1990 and 2014 (BOD by 61% nitrogen by 32% and phosphorus by 44%) and improved the quality of bathing sites across the EU (SWD, 2019). Nonetheless, EU waters fail to achieve good status under the Water Framework Directive and the inappropriately treated urban wastewater has been still pointed as an area for improvement. Additionally, there is growing evidence that contaminants of emerging concern are not targeted and are continuously discharged to the environment, even via appropriately treated wastewater. Especially pharmaceuticals, microplastics, human-related bacteria and antimicrobial resistance are recognized as a global threat (Everaert et al., 2020; Marano et al., 2020; Roca et al., 2015), however activated sludge-related bacteria are also disseminated. Their potential of participation in C, P, and N cycling in the receiver requires attention. Nitrification and denitrification are one of the most frequently investigated wastewater treatment processes. The abundance of bacteria responsible for these processes can be quantified using specific genes as molecular markers: *amoA* and *nxrA* genes for nitrifiers, and *nirK*, *nirS* and *nosZ* for denitrifying bacteria (Huang et al., 2019; Tang et al., 2020; Wang et al., 2014; Zhang et al., 2019).

This study covered two largest municipal WWTPs (WWTP-W and WWTP-D) located upon the Baltic Sea in northern Poland. They receive wastewater generated by a relatively large metropolitan area of Tricity (over 1 mln inhabitants, area around 400 km^2), with various branches of industry. In this study, it has been hypothesized that treated wastewater disposal can shape the microbial community of the recipient, especially by discharge of human related bacteria, washout of activated sludge community and release of functional N-cycling genes, increasing the nutrient cycling potential of the receiver. A wide range of complementary microbiological methods were applied to (1) investigate the year-round fluctuations in the microbial community of the WWTP effluent and (2) to elucidate the impact of its discharge on the marine waters. Microscopic observations and analysis provided information about prokaryotic cells abundance and morphology, cultivation on selective media enabled fecal bacteria enumeration, Next Generation Sequencing revealed the taxonomic composition of the microbial community and quantitative PCR gave the information about the abundance of nitrogen-related genes and provided the insight into the nutrient cycling potential of both the WWTP effluent and its recipient.

2. Materials and methods

2.1. Study area, sampling, and WWTP characteristics

The 24 h composite samples of the influent and final effluent were collected from the two WWTPs (WWTP-W and WWTP-D, Fig. 1). Both WWTPs operate on conventional mechanical and biological treatment with advanced nutrient removal followed by secondary settling tanks with activated sludge recirculation. Detailed WWTPs characteristics are presented in schematic technological diagrams in Fig. 1 and Supplementary Table S1. Influent and effluent samples (10 L) were collected twice a week for two years (from January 2012 to December 2013), transported to the laboratory in cooler boxes and immediately analyzed (Gerbl et al., 2014; Kim et al., 2011; Li et al., 2012; Throbäck et al., 2004).

Both WWTPs discharge treated wastewater into the Gulf of Gdansk (Natura 2000 area), approx. 2.3 km from the coastline at a depth of about 8 m via submarine collectors equipped with diffusers (Fig. 1). Samples of

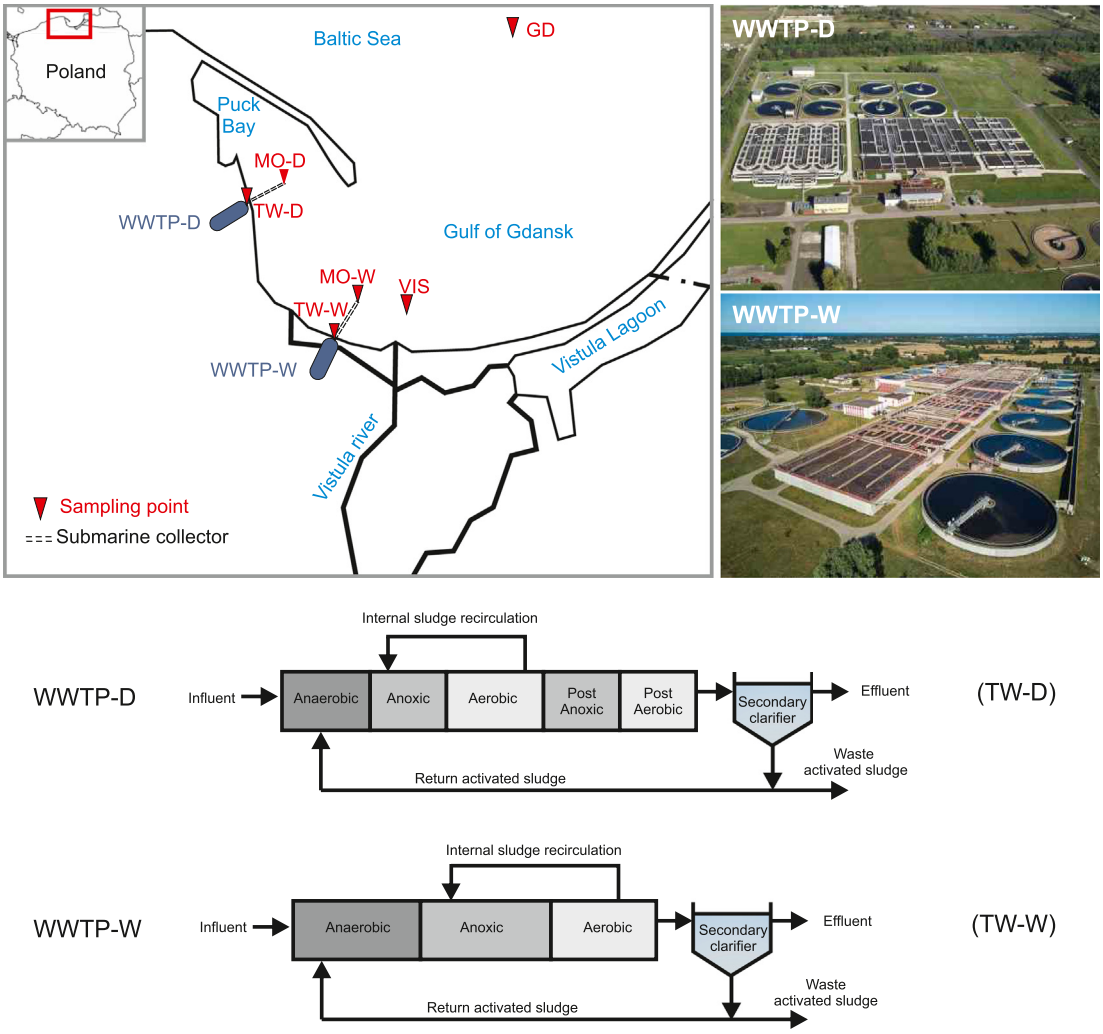

Fig. 1. Schematic technological diagrams, aerial photos and location of two WWTPs: Gdansk-Wschod (WWTP-W) and Gdynia-Debogorze (WWTP-D). Samples of treated wastewater were taken from both WWTPs (TW-W and TW-D, respectively), together with their marine outfalls into the Gulf of Gdansk (MO-W and MO-D, respectively) and two reference points – Vistula River estuary (VIS) and Gdansk Deep (GD).

the marine waters at the point of treated wastewater discharge (marine outfalls: MO-W and MO-D, respectively to the name of each WWTP) were sampled three times (August and September 2012, February 2013). Additionally, two reference points: Vistula River estuary (VIS) and Gdansk Deep (GD) were sampled twice (summer 2012 and winter 2012/2013). Vistula River was chosen due to the ecological importance of its flow on the Gulf of Gdansk quality. It is the longest river in Poland (1047 km) as well as in the area of the Baltic Sea, with its catchment equal to 194,424 km^2 (87% in Poland). The Vistula flows directly into the Gulf of Gdansk through a straight, man-made outlet, with an average annual flow of about 1×10^3 m^3/s at the mouth. Gdansk Deep (GD) is located in the open sea and is assumed to be isolated from the anthropogenic impact (Maksymowska et al., 2000). All environmental samples (points MO-W, MO-D, VIS, and GD) were collected with a Niskin bottle and transferred to sterile polyethylene bottles. The bottles were rinsed with the sampled water three times before sample collection. Samples were immediately transported in the cooler box at +4 °C to the laboratory.

2.2. Chemical analysis

Chemical analyses were conducted in all influent and effluent samples (twice a week for two years). Parameters were determined according to the Standard Methods (APHA, 2005): total nitrogen (TN), ammonium nitrogen ($N\text{-}NH_4$), nitrate nitrogen ($N\text{-}NO_3$), total phosphorus (TP), phosphate phosphorus ($P\text{-}PO_4$) and chemical oxygen demand (COD) were analyzed by a XION 500 spectrophotometer (Dr. Lange, GmbH, Germany); 5-day biochemical oxygen demand (BOD_5) – by a manometric respirometric BOD OxiTop® method; total suspended solids (TSS) – by a gravimetric method.

2.3. Microbiological analysis

Microbiological analyses of treated wastewater samples were conducted once a month, from June 2012 to May 2013 (12 samples per WWTP). Additionally, 10 environmental samples were collected: from marine outfalls of WWTP effluents (MO-D and MO-W, three times each), Vistula estuary (VIS, sampled twice), and Gdansk Deep (GD, sampled twice).

2.3.1. Microscopic methods

Direct Epifluorescent Filter Technique (DEFT) was used for the microscopic analyzes: DAPI staining (Porter and Feig, 1980) and Live/Dead assay (Boulos et al., 1999). Samples for microscopic enumeration with use of DAPI staining (50 mL) were fixed with particle-free buffered formaldehyde (Merck, Germany) to a final concentration of 2% and stored at +4 °C until the analysis. Subsamples of treated wastewater (0.5 mL) and marine waters (2 mL) were, stained with DAPI fluorescent dye (4′,6-diamidino-2-phenylindole, Thermo Fisher Scientific, US) to final concentration 1 μg mL^{-1} and filtered on black Nucleopore polycarbonate filters (Millipore Membrane Filter, 0.2 μm pore size, Merck, Germany). Filters were mounted

on a microscopic slide with non-fluorescent oil (Citifluor AF2: Agar Scientific, US) and stored at −20 °C until analysis. Microscopic slides were analyzed in triplicates under an epifluorescence microscope Nikon Eclipse 80i under 1000-fold magnification (HBO-103 W high-pressure mercury burner, Osram GmbH, US, 330–380 nm excitation filter, 420 nm barrier filter, 400 nm dichroic mirror). Triplicates of 10 microscope view fields, with a maximum of 60 thousand objects, were digitized using Nikon DS-5Mc-U2 high-resolution color digital camera and NIS-Elements BR 3.0 software. Image system analysis (MultiScan, v.14.02) with modification of Świątecki (1997) was applied to determine total prokaryotic cell number (TCN), prokaryotic biomass (PB), and average cell volume (ACV). Bacterial biomass was calculated using conversion factor (170 f. C μm^3) by Norland (1993).

Live/Dead assay was performed immediately after delivery to the laboratory on 0.5 mL treated wastewater and 2 mL marine water subsamples, stained in duplicates with fluorescent dyes SYTO9 and PI from the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA). Identical volumes of each dye were applied (0.1 mL), samples were incubated in darkness for 30 min and filtered through 0.2 μm polycarbonate filters (Whatman, UK). Filters were kept at -20 °C until further examination. The percentage of alive cells was determined using an epifluorescence microscope (400–440 nm and 450–490 nm excitation filters, 455 nm and 505 nm dichroic mirror, 470 nm and 520 nm absorption filter) under 1000-fold magnification. Green fluorescence (excitation/emission: ~495 nm/~515 nm) corresponds to live bacteria with undamaged cell membrane, while damaged (dead) cells produce a bright red fluorescence (excitation/emission: ~495 nm/~635 nm). The bacteria in 2 repeats of 10 fields were counted and the result is given as a percentage of live cells in all observed cells.

2.3.2. Cultivation methods

In this study, Gram-negative enteric rods from the *Enterobacteriaceae* family were enumerated, including indicators of fecal contamination, such as fecal coliforms and *Escherichia coli*. Cultivation was performed immediately after sample delivery to the laboratory. Serial dilutions were applied: 10^{-4} to 10^{-1} mL for wastewater, and 10 to 500 mL for marine waters was filtered in triplicates on cellulose membrane filters (47 mm diameter, 0.45 μm pore diameter, Whatman, UK). Bacteria were grown on Chromocult® Coliform Agar and Membrane Fecal Coliform Agar (Merck, Germany) according to the manufacturer specifications, as is summarized in detail in Supplementary Table S2. Based on bacterial colony growth, the amount of colony-forming units (CFU) per 100 mL was determined.

2.3.3. DNA extraction and bacterial 16S rRNA gene amplification

Subsamples of treated wastewater (100 mL) and marine waters (400 mL) were filtered on polycarbonate filters (0.2 μm pore diameter, Merck, Germany) and stored at −80 °C for the DNA extraction. Duplicates of the filtered material for each sampling point were merged for DNA extraction and considered as one sample in further taxonomic analysis. The total community DNA was extracted and purified using Genomic Mini AX Bacteria + (mod.5) isolation kit (A&A Biotechnology, Poland) and quantified by spectrophotometry at 260 nm using Nanodrop (Thermo Fisher Scientific, UK). The presence of bacterial DNA was confirmed by real-time PCR with SYBR Green fluorochrome, in Mx3000P thermocycler (Stratagene, USA). The following PCR conditions were used: initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of denaturation (95 °C for 15 s), annealing (58 °C for 30 s), fluorescence measurement, and extension (72 °C for 30 s). For amplification of 16S rDNA fragments, the universal primers were applied: 1055F and 1392R (Ferris et al., 1996). The final check on the DNA quality was done by determination of the PCR product melting curve and measuring fluorescence at temperatures from 65 °C to 95 °C. The PCR products were stored at -20 °C for sequencing.

2.3.4. Sequencing, taxonomic assignment, and data analysis

To establish microbial community composition of the analyzed samples, 16S rRNA gene V3-V4 region amplified with 341F and 785R primers pair (Klindworth et al., 2012). Paired-end sequencing was performed with an Illumina MiSeq 2 × 300 bp platform by the Macrogen company (Macrogen Inc., South Korea) and following manufacturer's run protocols. FASTQ files were generated from MiSeq Reporter (Illumina) and the paired reads were initially joined with the FASTQ joiner and subjected to quality control with the FASTQC (at quality cut-off value = 20). Tools are available at the UseGalaxy server (https://usegalaxy.org). Sequences shorter than 120 bp were excluded from further analysis. Classification of the reads on each taxonomic level was carried out with Silva NGS server (http://www.arb-silva.de) by the use of database release version 138 at the species similarity level of 90% and OTUs (operational taxonomic units) clustering at 97%. Alpha diversity was assessed based on richness and diversity indices: Chao1, Shannon (H′) and Simpson (D), obtained in CLC Genomics Workbench software.

2.3.5. Quantitative PCR of nitrogen-cycling-related genes

Quantitative real-time PCR was used to validate the absolute abundance of 16S rRNA and some functional genes, including *amoA*, *nxrA*, *nirS*, *nirK,* and *nosZ*. The fragments coding these genes were amplified with specific primers listed in Table S3 (Supplementary Materials). Amplification of real-time PCR products was carried out with Stratagene Mx3000P thermocycler (Agilent Technologies, US) using SYBR Green as a detection system in a reaction mixture of 20 μL containing: 0.1 μL of each *nirS* and *nirK* primers, 0.4 μL for *nxrA* primers and 1 μL for *amoA* and *nosZ* primers; 10 μL of Real-Time 2xRT-PCR Mix SYBR A mixture (A&A Biotechnology, Poland), 1 μL of DNA diluted template corresponding to 10 ng of total DNA, and Rnase-free water to complete the 20 μL volume. All primer pairs amplifying gene fragments of *nirS* and *nirK* were run with an initial denaturation of the DNA at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. For *amoA*, *nxrA,* and *nosZ* a similar procedure was applied, with the only difference in primer annealing temperature (55 °C, 63 °C, and 65 °C, respectively).

2.4. Statistical analysis

PCA was performed in R software (version 3.6.2), using the FactoMineR package, on the scaled dataset. Canonical correspondence analysis (CCA), combining the basic properties of a typical correspondence analysis with those of a constrained ordination, was performed with the vegan package (Dixon, 2003). To observe the clusters of microbial taxa, ordered along the canonical axes, following their ecological optima, and to obtain a clearer model with a limited number of significant axes, a forward selection of explanatory variables was performed for each WWTP. To identify the explanatory variables that significantly explained variation in microbial communities, forward selection was performed using the ordistep function within the vegan package (999 Monte Carlo permutations, alpha <0.05). For transparency, only taxa of the relative abundance over 3% at the family level in at least one sample were used for CCA analysis. For the heatmap with dendrogram, a hierarchical clustering was performed using the average (UPGMA) method on the Bray-Curtis dissimilarity matrix, evaluated on the reduced dataset.

3. Results and discussion

WWTPs tested in this study (WWTP-W and WWTP-D) are located near each other, serve similar urban catchments (equipped with a sanitary network) and perform enhanced simultaneous C/N/P removal (for details see Materials and Methods). Both also discharge the effluent into the coastal marine waters via marine outfalls (MO-W and MO-D, respectively). To better elucidate the characteristics of coastal areas impacted by treated wastewater (MO-W and MO-D), and non-impacted points were tested: Vistula River estuary (VIS) and Gdansk Deep (GD). Microbiological results were supported by physical and chemical parameters.

3.1. Chemical parameters

The wastewater profiles depend on many factors, e.g.: catchment size and type of sewer network, number of people served, and industrial

discharges (Deblonde et al., 2011). This study focused on two WWTPs (WWTP-W and WWTP-D), which treat wastewater generated by a metropolitan area of Tricity (Fig. 1). Chemical parameters (COD, BOD_5, TSS, TN, $N\text{-}NH_4$ and TP) in raw wastewater (Fig. 2a) were typical for the studied urban catchment (Krzeminski et al., 2012; Pasztor et al., 2009) and indicated high similarity between WWTPs (Supplementary Fig. S1A). Factors

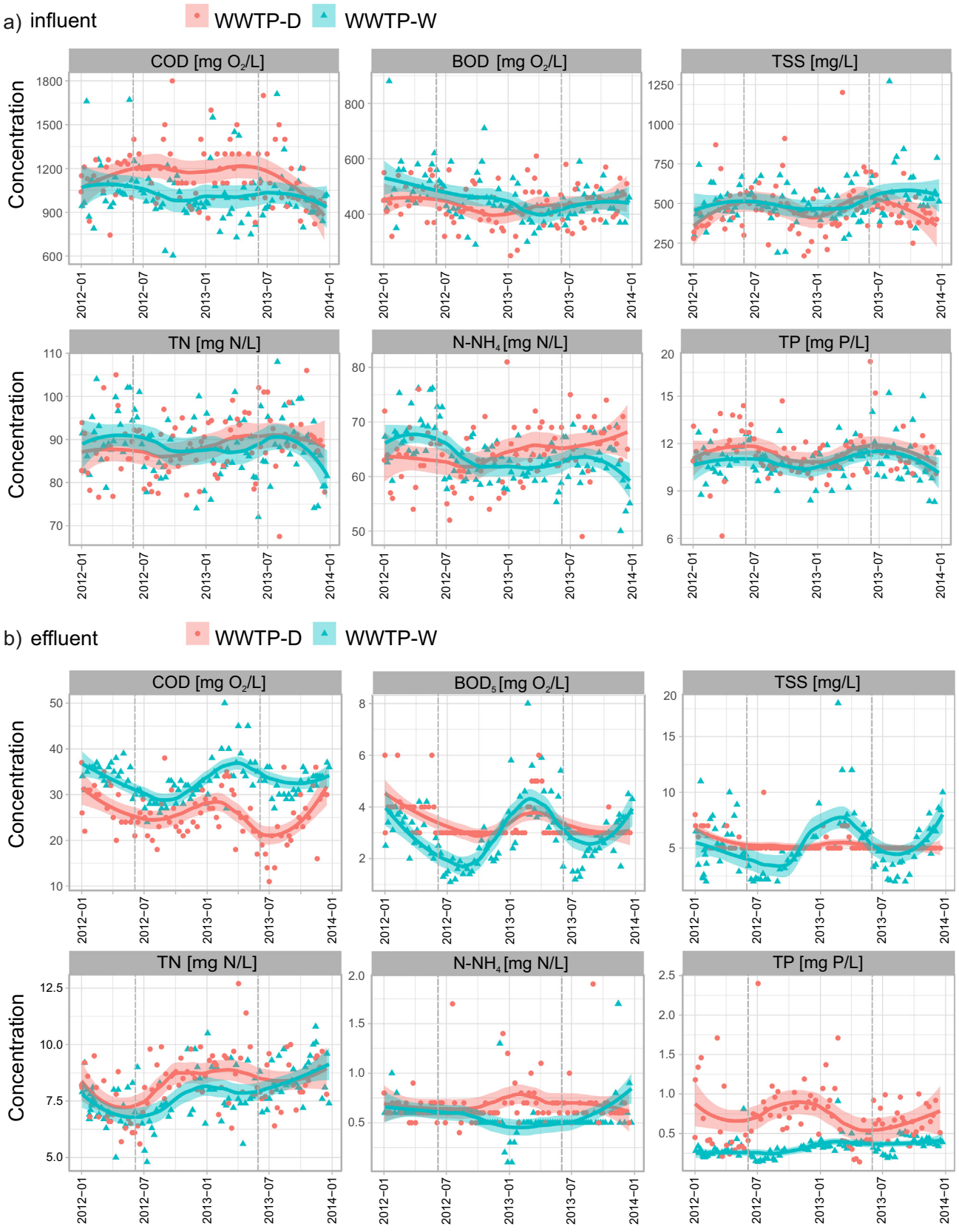

Fig. 2. Time series of basic chemical parameters in a) influent and b) effluent for both WWTPs (WWTP-D and WWTP-W) investigated in this study.

explaining the variability of raw wastewater were most importantly: TP, TN, and N_{org} (organic nitrogen) for WWTP-D, and COD, TSS, and TN for WWTP-W (Supplementary Fig. S1B and C).

Higher concentrations of the chemical parameters at the influent did not however always result in the higher concentration in the effluent (Fig. 2a, b). The principal component analysis (PCA) revealed the seasonality pattern of decreasing wastewater treatment efficiency in winter months (Fig. 3a, c), as PC1 correlates positively to the ambient temperature variability and negatively with BOD and TSS in treated wastewater (Fig. 3a). This trend was also shown in other studies (Xue et al., 2019). PC2 reflects mostly treated wastewater parameters and it strongly separates the treatment plants (WWTP-W and WWTP-D) from each other (Fig. 3b). The most important factors separating the WWTP effluents are nitrogen and phosphorus compounds concentrations and their corresponding removal efficiencies (Fig. 3a,b, Supplementary Fig. S2), which may result from the differences between applied treatment systems, WWTPs size and operator's management.

Note that deterioration of treated wastewater quality is usually connected with an increase in WWTP effluent turbidity, and inter-related parameters such as mainly COD and BOD (Figs. 2b, 3a) were mainly linked to the turbulence in the activated sludge process. The reason may be a seasonal change of activated sludge community structure (see Section 3.3) due to the drop of both wastewater and ambient temperature. As a consequence, sedimentation disturbance and activated sludge biomass washout may occur and lead to deterioration of the treated wastewater quality, causing the need to change the wastewater treatment strategy. A common practice is to increase the density and age of activated sludge by prolonging biomass retention time (necessary to maintain an appropriate rate of autotrophic processes such as nitrification), while dosing of coagulants (e.g.: PIX/PAX) prevents biomass washout with treated wastewater (Boguniewicz-Zablocka et al., 2020). Nevertheless, despite preventive measures undertaken at both studied WWTPs, microscopic analysis indirectly confirmed dispersed biomass being washed out during the winter season (Section 3.2.1 and Fig. S3), observed especially between October 2012 and April 2013. This phenomenon was also confirmed by NGS analysis of TW-D and TW-W (for details see Section 3.3.1). Despite the seasonal deterioration of the TW-D and TW-W quality, all indicators of treated wastewater quality remained lower than current discharge limits (COD <125 mg O_2 L^{-1}, BOD <15 mg O_2 L^{-1}, TN < 10 mg L^{-1}, TP < 1 mg L^{-1}, TSS < 35 mg O_2 L^{-1}) (Dz.U. 2019 poz. 1311).

Marine outfalls of studied WWTPs (MO-D and MO-W) are located 2.3 km from the coastline (Fig. 1) to ensure proper dispersion of treated wastewater (TW-W and TW-D) in the receiver (Gulf of Gdansk and its internal part - Puck Bay) and to avoid the deterioration of coastal bathing areas. According to the obtained results, COD and BOD concentrations in TW-D and TW-W were similar to these noted in environmental samples (VIS and GD) during the summer (Supplementary Table S4), but the other parameters (TN, N-NH_4 and TP) were consistently higher. These results confirm continuous supply of nutrients from WWTPs to the marine waters via marine outfalls (MO-D and MO-W). Interestingly, at MO-D and MO-W all the tested chemical parameters were on a similar or lower level than noted at Vistula estuary (VIS) (Supplementary Table S4). This can be explained by the Vistula estuary geomorphological and hydrological features, which receive on average 1081 m^3/s discharge from the Vistula River (Wielgat-Rychert et al., 2013). Because Vistula River serves as wastewater receiver and its catchment area is intensively cultivated cropland, its estuary is known to receive high nitrogen and organic matter loads: 97000 t TN yr^{-1} and 600,000 t C yr^{-1}, respectively (Bartl et al., 2019; Maksymowska et al., 2000). This leads to high concentrations of nutrients and organic matter (Pastuszak et al., 2012), high primary production rates (Witek et al., 1999; Wielgat-Rychert et al., 2013), and thus to the eutrophied state (Maksymowska et al., 2000). On the other hand, high riverine nitrogen loads are known to increase rates of microbial processes (Seitzinger et al., 2006), which was confirmed by the level of N-cycling genes in VIS, similar as for MO-D and MO-W (for details see Section 3.4).

3.2. Enumeration of bacteria

3.2.1. Microscopic analysis

Availability of nutrients and increased temperature of the wastewater are the conditions supporting bacterial growth. Therefore, as suspected, all the parameters tested under the microscope (TCN, PB, ACV, and Live/Dead) were on average higher in the treated wastewater (TW) than in the environmental samples: MO, VIS and GD (Fig. 4a, b). TCN and PB values in the treated wastewater were similar for both WWTPs (average 2.22 mln cells/mL and 69.22 μg C/mL for TW-D, 2.21 mln cells/mL and 68.27 μg C/mL for TW-W). They ranged between 1.65 and 2.91 mln cells/mL and 30.6–80.5 μg C/L, what is the same magnitude as observed in other WWTP effluents (Bray et al., 2021, Kalinowska et al., 2020).

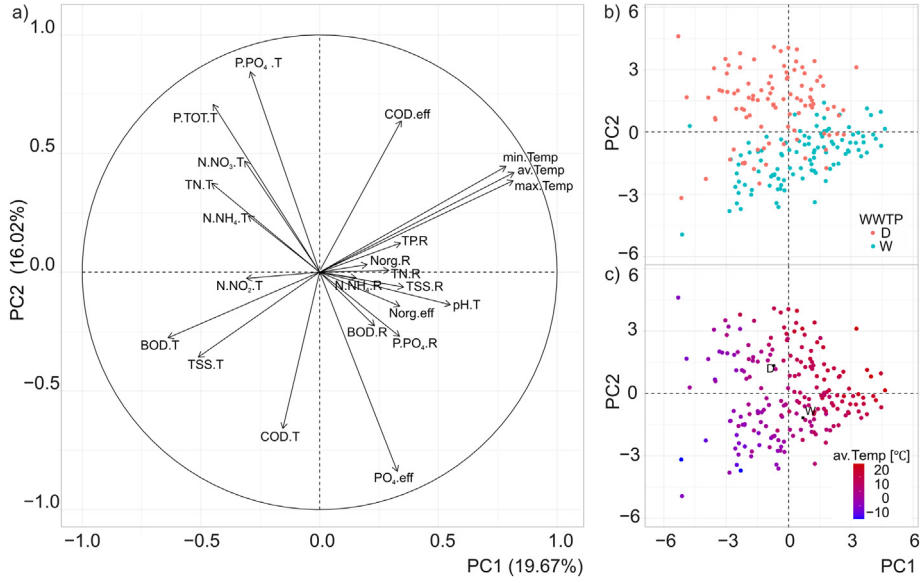

Fig. 3. Principal component analysis (PCA): (a) loading plot, and score plots: (b) sampling colored by the WWTP supplementary categorical variable, (c) sampling colored by the average ambient temperature variable. Analysis was conducted on the entire dataset constituted by the chemical parameters of both raw and treated wastewater (indicated by *R* or *T* after the variable name), and relevant removal efficiencies (indicated by *eff*).

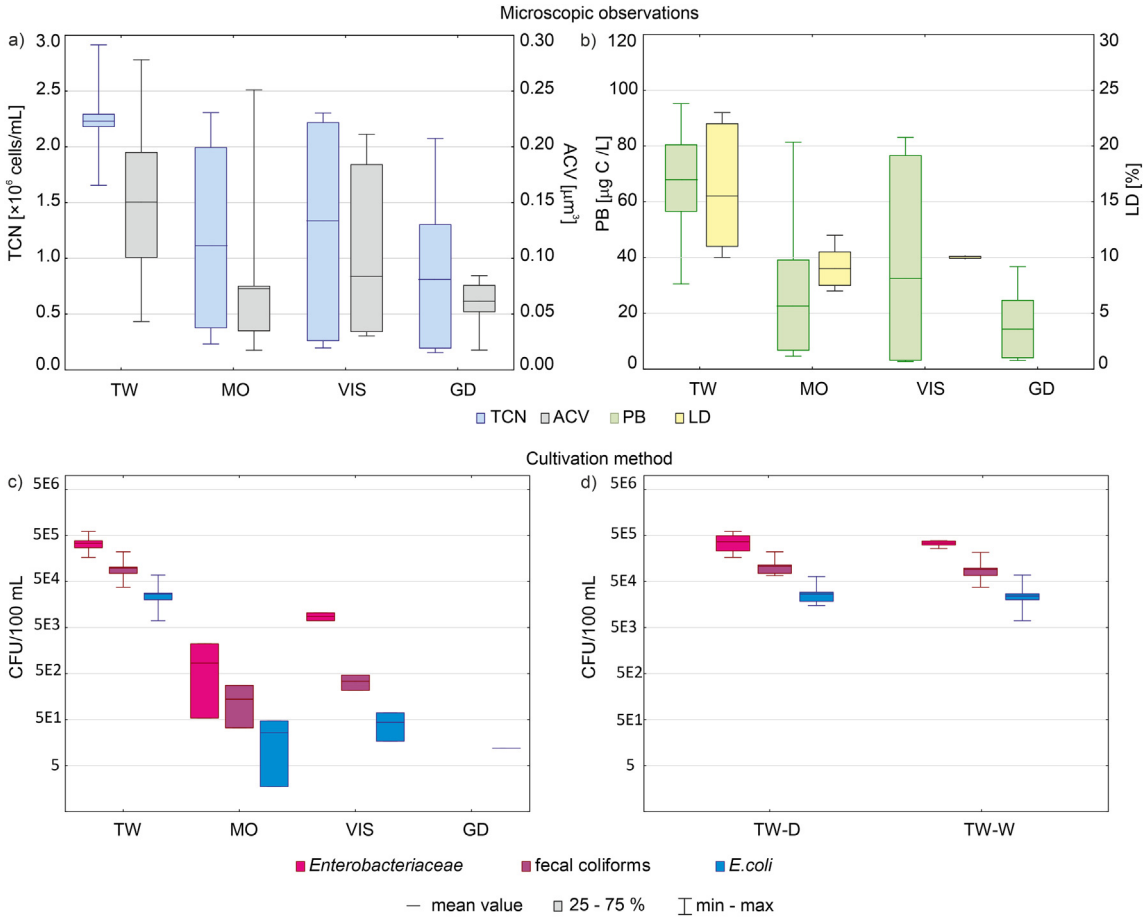

Fig. 4. Results of the microscopic and cultivation analysis for treated wastewater (TW), its marine outfalls (MO), Vistula estuary (VIS), and Gdansk Deep (GD): a) total prokaryotic cell number (TCN) and prokaryotic biomass (PB) obtained in DAPI staining; b) average cell volume (ACV) obtained in DAPI staining and percentage of alive bacteria obtained in Live/Dead assay (LD; not carried out for GD samples); c) sanitary indicators in various sample types, d) comparison between tested WWTPs effluents (TW-D and TW-W). *Enterobacteriaceae* and fecal coliforms were not tested for GD. The abundance of various bacterial groups is expressed as a number of colony forming units (CFU) in 100 mL.

Direct microscopic observations revealed some seasonal variability in the bacterial morphology in the treated wastewater. During the summer, the bacterial cells observed in TW-D and TW-W were rather larger and free-swimming, while in winter more numerous, smaller, and structured into small flocks (Fig. S3). It was already suggested that dispersed biomass in activated sludge expresses different aggregative properties than the biomass of settleable sludge. Additionally, factors that may negatively impact the production of extracellular polymeric substances (EPS) or may cause EPS destruction also influence the presence of floc-forming species. EPS are the structural backbone of the activated sludge flocs, and play a crucial role in activated sludge flocculation, settling, and dewatering. Note that EPS acts also as a survival mechanism for bacteria, protecting them from stress conditions, such as dehydration, presence of toxic substances, or nutrient deficiency (Laspidou and Rittmann, 2002). EPS destruction may be caused by turbulence in the activated sludge biomass, but technological processes and other factors important in this phenomenon are still not fully understood.

Environmental samples were characterized by larger fluctuations of TCN and PB than in the WWTPs' effluents, as wastewater is more stable in terms of temperature and availability of nutrients. The amount of prokaryotic cells varied between 0.18 and 2.30 mln cells/mL at the in marine outfalls, 0.19–2.30 mln cells/mL at Vistula estuary and was the lowest (0.15–2.07 mln cells/mL) at Gdansk Deep (Fig. 4a, b) what is in the range noted in the other studies from this Baltic Sea area (Piwosz et al., 2013, Kudryavtseva et al., 2012, Ameryk et al., 2014). Prokaryotic biomass followed the same pattern and ranged between 3.7–81.5 µg C/L for MO, 2.5–82.8 µg C/L for VIS and 2.9–36.5 µg C/L for GD. Large fluctuations of microbial microscopic parameters in the marine samples result from sampling in warm (August, September) or cold period (November, February) and they correspond to the general seasonal trend confirmed by other authors (Ameryk et al., 2021; Freese et al., 2006; Piwosz et al., 2013). Gulf of Gdańsk is an eutrophicated water reservoir, rich in phyto- and bacterioplankton during the vegetation period from April to October (Witek et al., 1997) and several studies (Danovaro and Fabiano, 1997; La Ferla et al., 2014, 2010) indicate that the availability of nutrients affects the bacterial parameters. Both terrigenous organic matter and autochthonous matter of phytoplankton origin can also support the growth of heterotrophic bacteria (Ameryk et al., 2005). The lower ambient temperature and limited nutrient availability in the marine waters during winter were followed by one magnitude lower bacterial cell abundance and biomass (February) than in the summer season (August, September). Also the Vistula River introduces nutrients to the Gulf of Gdansk, therefore it stimulates the bacterial production in its internal part (Ameryk et al., 2005; Wielgat-Rychert et al., 2013) what is reflected by both high values and large fluctuations in the microscopic parameters at VIS. Gdansk Deep (GD) was characterized by the lowest values of all microbial parameters (Fig. 4a, b, c), which supports its choice as the reference point, being under the limited impact of anthropogenic and riverine origin.

Results of the Live/Dead assay showed that treated wastewater contained a higher ratio of the live cells than the environmental samples (Fig. 4b). This was according to expectations, as activated sludge can contain up to 80% of live and active cells (Kocwa-Haluch and Woźniakiewicz, 2011)

which typically occur in sludge flocs. Only about 10% of activated sludge biomass tends to remain in suspension and/or to detach easily from average sludge flocs. Thus, observed increased Live/Dead ratio in treated wastewater may indicate a poor sedimentation and biomass washout. In environmental samples, the share of active bacterial cells was not exceeding 15%, and the highest values were observed at marine outfalls (MO-D and MO-W). The quantification of alive bacteria is important in the context of microbial production, organic matter decomposition, and for assigning microbial activities to individual organisms (Kogure et al., 1979; Rodriguez et al., 1992; Schumann et al., 2003). Microscopic techniques, including Live/Dead assay, were in recent years superseded by metagenomic methods and currently are rarely used in environmental studies. However, these methods still provide significant information on cell viability, which is missed or biased when using methods based on DNA approach (Cangelosi and Meschke, 2014; Guo and Zhang, 2014; Li et al., 2017; Nielsen et al., 2007; Nocker et al., 2010).

3.2.2. Bacteria cultivation

The bacterial community can be assessed by a variety of approaches. The culture-dependent methods have been in recent years dislodged by the high-throughput sequencing of the 16S rRNA gene due to less time-consuming procedure, ability to generate larger datasets and improved access to the rare biosphere (Tytgat et al., 2014). However, the cultured organisms from a given sample might be important for determining some impacts, such as e.g.: anthropogenic stress on indigenous microbial communities. Moreover, short 16S rRNA gene reads lead to technical limitations to obtain species-level identification (Bibby et al., 2010; Ju et al., 2016; Luo and Angelidaki, 2014; Ye and Zhang, 2011). Thus in this study, the indicators of fecal contamination were additionally assessed by cultivation of gram-negative enteric rods from *Enterobacteriaceae* family together with the fecal coliforms and *E. coli* (Fig. 4c and d).

An average number of *Enterobacteriaceae* was similar in both WWTPs effluents (3.6×10^5 CFU/100 mL for TW-D and 3.3×10^5 CFU/100 mL for TW-W, Fig. 4d), but values in TW-W were more uniform throughout the year (from 2.6 to 3.8×10^5 CFU/100 mL versus from 1.7 to 6.1×10^5 CFU/100 mL in TW-D, Fig. 4d). No clear seasonal pattern was observed in terms of their variability. In environmental samples both *Enterobacteriaceae* and *E. coli* were on average three orders of magnitude lower than in WWTP effluents. Interestingly, *Enterobacteriaceae* were ten times less abundant at MO than at VIS (8.4×10^2 CFU/100 mL versus 8.7×10^3 CFU/100 mL) and not detected at GD (Fig. 4c).

Fecal coliforms, as well as their representative - *E. coli,* were detected also in environmental samples and presented a similar trend as the *Enterobacteriaceae* family: their average values were higher for the Vistula River estuary than the marine outfalls of the treated wastewater, but Gdansk Deep presented the lowest abundance of these bacteria. The number of *E. coli* varied from 1.5×10^4 to 6.4×10^4 CFU/100 mL in TW-D and from 7.0×10^3 to 6.9×10^4 CFU/100 mL in TW-W, respectively (Fig. 4d), which is rather typical for treated wastewater (Łuczkiewicz et al., 2010; Marano et al., 2020). It was also reported by others that fecal bacteria, even if removed with high efficiency of over 90%, are still released to the recipient with WWTP effluent as a result of their high initial number (Lucena et al., 2004; Marano et al., 2020). However, in none of the environmental samples the number of *E. coli* exceeded the allowable standard for bathing sites (<500 CFU/100 mL), according to the New Bathing Water Directive (2006/7/EC). Previous studies of Polish rivers receiving treated wastewater also reported presence of fecal coliforms and *E. coli* in Vistula (Donderski and Wilk, 2002; Walczak, 2008) or other rivers (Bączkowska et al., 2022, 2021; Niewolak, 1998). Their abundance was in the similar range as presented in this study and very likely was supported by high availability of easily absorbed organic matter (Donderski and Wilk, 2002).

3.3. Metagenomic analysis of microbial community

Beyond laboratory-grown cultures, metagenomic tools have significantly enhanced our understanding of microorganisms associated with numerous habitats. In this study microbial composition of WWTP effluents (TW-D and TW-W) and environmental samples impacted (MO-D and MO-W) and not directly impacted by treated wastewater (VIS and GD) were analyzed (Fig. 5a,b,c). A total of 6600 OTUs were identified from 2,726,599 sequences (average length of 301 bp) obtained from 34 samples (24 samples of treated wastewater collected from June 2012 to May 2013 - 12 for each WWTP; and 10 environmental samples). Alpha diversity was quantified using three richness and diversity indices: Shannon, Simpson and Chao1 (Fig. 5d). On average, they were the highest for treated wastewater and lower for the environmental samples, and for Shannon and Simpson the differences between these sample types were significant. WWTPs did not differ significantly from each other, but throughout the year the microbial community composition of the WWTP effluent fluctuated (Fig. 6a, b) and its biodiversity decreased in winter, what reflects the trend found in the activated sludge reactor (Wang et al., 2016).

For an open-sea sampling station GD, the alpha diversity was the lowest, with the exception of Chao1, which is highly influenced by the presence of rare taxa. Samples being under treated wastewater impact (MO) presented higher microbial diversity, what has been also noted in other studies (García-Armisen et al., 2014; Kalinowska et al., 2020; Price et al., 2018; Wakelin et al., 2008). This supports the hypothesis of increasing biochemical potential of the natural waters due to WWTP effluent discharge. Nevertheless, these changes may be temporary and may depend highly on the water mixing (Price et al., 2018). The share of treated wastewater in recipients may also play a significant role. Increased biodiversity indices for VIS may reflect the massive amounts of river waters introduced by the Vistula River to the Gulf of Gdansk (approx. 30 km^3 annually) and therefore the intensive mixing of marine and freshwater communities in the river estuary.

Taxonomy-based analysis indicated that *Bacteria* constituted a majority of the total microbial community, and *Archaea* less than 0.2% in a single sample (higher share in treated wastewater, while in environmental samples maximum 0.02%). *Archaea* were represented mainly by *Parvarchaeota*, class *Parvarchaea*, but smaller shares of *Crenarchaeota* and *Euryarchaeota* (*Methanobacteria* and *Methanomicrobia* classes) were also found, however only in TW-D and TW-W, which is in agreement with other wastewater studies (Gonzalez-Martinez et al., 2018; Greay et al., 2019; Tiirik et al., 2021). Among 56 identified bacterial phyla, 38 were present in minor shares (less than 1% in each sample). Fig. 5 shows the relative abundances of the most abundant phyla and 11 of them were common for all the samples analyzed, however their abundance varied significantly. They belonged to *Actinobacteria* (4.5–41.0%), Proteobacteria (5.5–56,9%), Bacteroidetes (0.5–12.4%), Firmicutes (0.03–16.6%), Verrucomicrobia (0.1–9.7%), and Planctomycetes (0.04–8.5%), with smaller shares of Acidobacteria (<0.01–1.3%), Chlamydiae (<0.01–2.3%), (0.01–3.9%), Cyanobacteria (0.05–49.4%) and Saccharibacteria/TM7 (<0.01–60.9%). Heatmap with dendrogram (Fig. 5e) confirms the higher similarity among the environmental samples, clearly separated from TW samples.

3.3.1. Microbial community of WWTP effluents (TW-D and TW-W)

In the case of TW-D and TW-W samples, 22 phyla formed the core microbial community. Among them, the most abundant in TW-D and TW-W were *Proteobacteria* (up to 57% and up to 38%, respectively), *Saccharibacteria/TM7* (up to 48% and up to 61%, respectively), *Acidobacteria* (up to 33% and up to 41%, respectively), *Firmicutes* (up to 17% and up to 15%, respectively), *Parcubacteria/OD1* (up to 16% and up to 12%, respectively) and *Bacteroidetes* (up to 12% and up to 10%, respectively), Fig. 5a and b. Most of those taxa, were also identified as core ones in the activated sludge (Wu et al., 2019). Similarity of microbial communities detected in TW-D and TW-W samples can be explained by similar urban catchments and sanitary networks but most of all by treatment processes served by the tested WWTPs (enhanced simultaneous C/N/P removal; for details see Materials and methods). Although, it should be noted that similar microbial communities in treated wastewater were also shown by others (Cai et al., 2014; Do et al., 2019; García-Armisen et al., 2014; Tiirik et al., 2021; Xue et al., 2019). For instance, Do et al. (2019), who tested WWTPs effluents in Ireland, reported among predominant phyla:

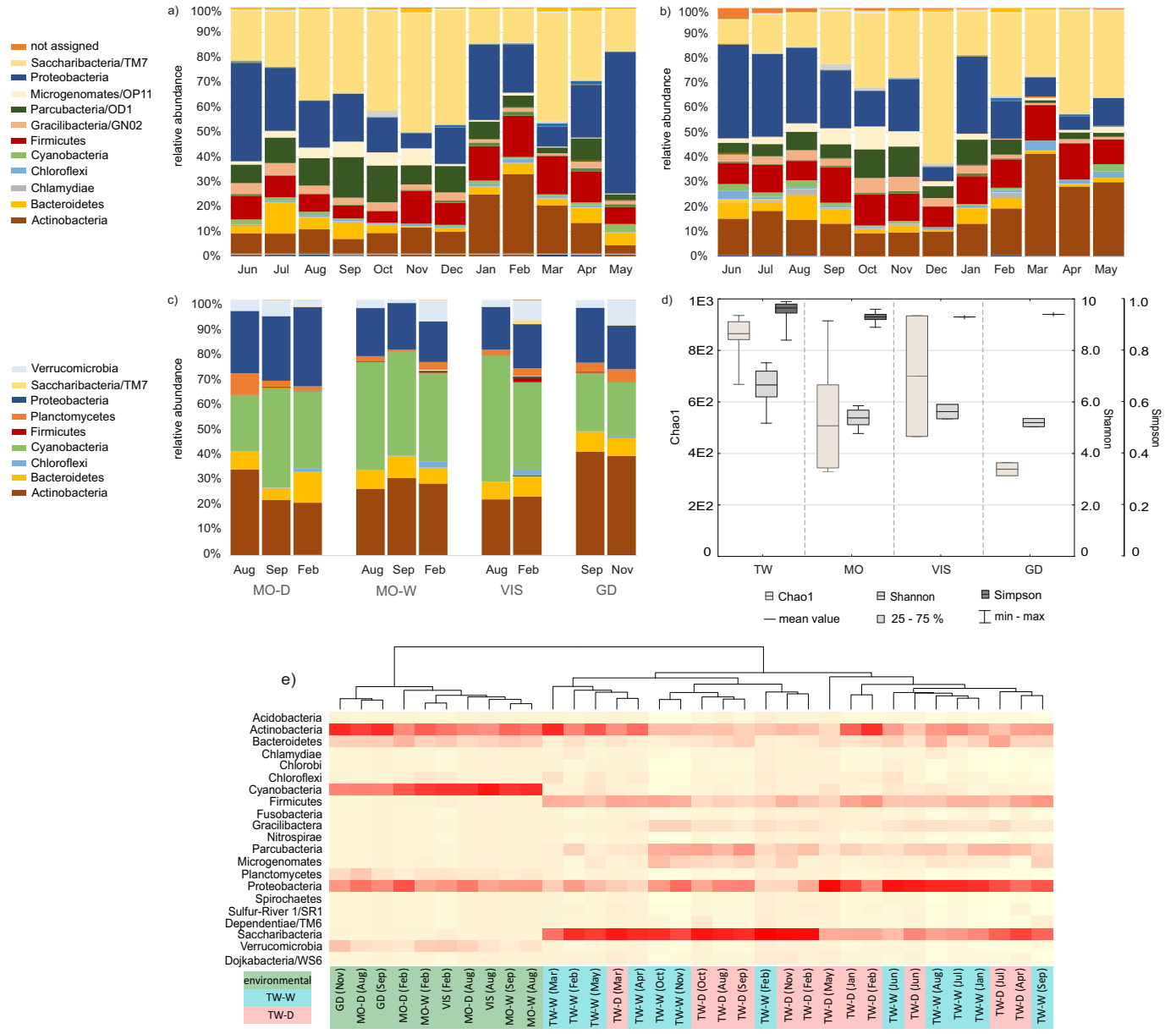

Fig. 5. Taxonomic relative abundances at phylum level noted in WWTP effluents TW: a) TW-D and b) TW-W and in c) environmental samples of marine outfalls MO (MO-D, MO-W), Vistula estuary (VIS) and Gdansk Deep (GD). Names and colors are listed for phyla with relative abundance >3% in at least one sample. Fig. 5d presents Chao1, Simpson, and Shannon indices for the sample types: TW, MO, VIS and GD. Fig. 5e shows heatmap of main phyla with the dendrogram of the samples.

Proteobacteria (67%), *Actinobacteria* (up to 50%), followed by *Bacteroidetes* (up to 18%) and *Firmicutes* (up to 16%). Similar phyla, but in different share were noted in the outflows of WWTP located in Belgium (García-Armisen et al., 2014): *Proteobacteria* (up to 74%), followed by *Bacteroidetes* (up to 37%) and *Actinobacteria* (up to 18%). In WWTP from Hong Kong (Cai et al., 2014) the following share of phyla were found: *Proteobacteria* (up to 60%), *Saccharibacteria/TM7* (up to 25%), *Bacteroidetes* and *Acidobacteria* (up to 20%), followed by *Firmicutes* (up to 14%). The microbial community of treated wastewater has been rarely studied, especially in terms of seasonal variations in composition and diversity (Wang et al., 2016), therefore this issue is not fully recognized and understood. However, worldwide similarities observed until now at high taxonomic levels may suggest that the microbiome composition of WWTP effluents is to some extent consistent among WWTPs (Adrados et al., 2014; Cai et al., 2014; Silva-Bedoya et al., 2016). Wastewater treatment is intensively adjusted by operators in winter as a response to the current effectiveness of microbiological processes, particularly those linked to nitrogen and phosphorus removal. Thus, the metagenomic approach indirectly confirmed the seasonal disruptions of wastewater processes, already confirmed by elevated chemical parameters (see Section 3.1) and presence of numerous, small-structured flocks in TW-D and TW-W during the cold season (see Section 3.2.1, and Fig. S3).

Canonical correspondence analysis (CCA) analysis was done with regard to WWTPs' inflow and effluent chemical parameters and microbial communities in treated wastewater (TW) samples. For WWTP-D (Fig. 6a), four parameters were chosen as explanatory variables: concentration of organic nitrogen in the WWTP influent (N_{org}.R), P-PO_4 removal efficiency (PO_4.eff), TN/COD.T ratio in the effluent and ambient temperature, explaining overall 73.0% of the total variance. The winter samples (January–February) were characterized by the worst PO_4 removal efficiency, combined with the lowest organic nitrogen concentration in the influent and lowest temperature. Together with early spring samples (March–April) they contained increased amounts of bacteria potentially related to foaming and bulking (family *Microthrixaceae*, mostly *Candidatus Microthrix*), originating from human gut (*Carnobacteriaceae*

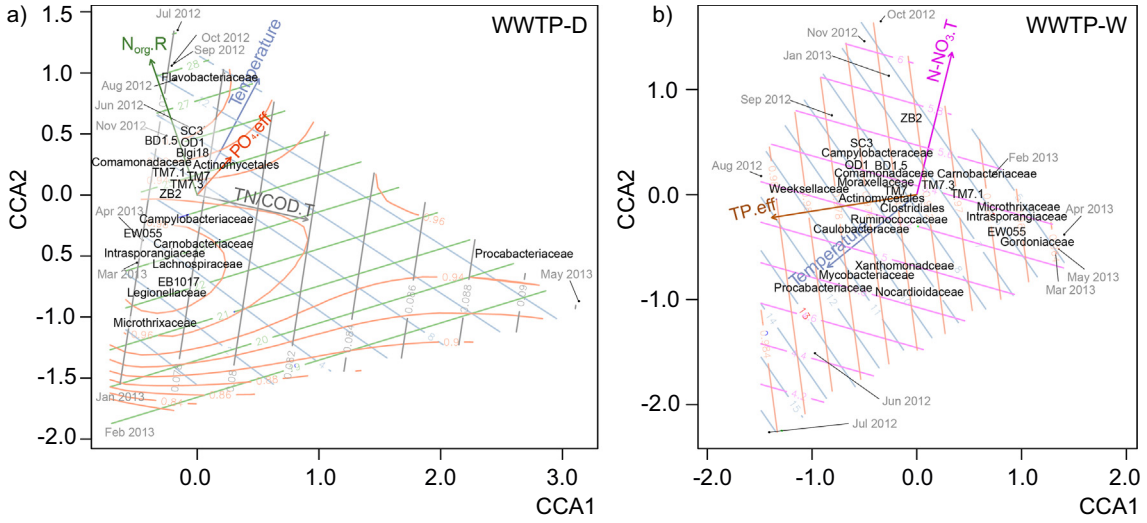

Fig. 6. Canonical correspondence analysis (CCA) in respect to chemical parameters and NGS analysis of samples from a) WWTP-D and b) WWTP-W. The arrows represent the explanatory variables and the lines of corresponding color show their values. Representative bacterial taxa are given in black and the sampling date is given in grey. From the microbial community, the representative taxa of the relative abundance over 3% at the family level in at least one sample were chosen for the analysis.

and *Lachnospiraceae*), or potentially pathogenic ones (*Campylobacteraceae* and *Legionellaceae*). *Campylobacteraceae* would be of special concern, as they were found to be positively correlated with occurrence of some antibiotic resistance vectors in the WWTPs effluents (mainly β-lactamases and integrons) (Fernandes et al., 2019).

The total variance for WWTP-W samples explained by CCA was lower than for WWTP-D and involved less variables: total phosphorus removal efficiency (TP.eff), nitrate nitrogen concentration in the effluent (N-NO_3.T) and ambient temperature (Fig. 6b). It showed the dominance of similar taxa in the WWTP-W effluent all over the year. Winter/spring samples were associated with presence of bacteria that imply treatment efficiency deterioration. In April and May, particularly high shares of *Gordonia* (an opportunistic human pathogen widely distributed in aquatic and terrestrial environments) were found. Summer/autumn samples, characterized by higher N-NO_3 concentrations contained microorganisms commonly found in activated sludge reactors (representatives of *TM7/Saccharibacteria* phylum, e.g. *EW055*, Gómez-Acata et al., 2017) or in environmental samples: anoxic (*ZB2* and other representatives of *Parcubacteria/OD1,* Harris et al., 2004), or potentially associated with mammal presence (BD1.5 - representatives of *Gracilibacteria/GN02*, Dudek et al., 2017). It is worth noting that in the case of both WWTPs, the summer-autumn samples were usually grouped together, while winter-spring were separated from them (Fig. 6a, b). For WWTP-W the samples from June–July were clearly separated from the other samples regarding both dominating microbial taxa, as well as chemical parameters (primarily N-NO_3 concentration).

During wastewater processes, nitrogen is usually removed via the nitrification-denitrification pathway. Most nitrifiers are strongly associated with activated sludge, therefore the structural integrity of flocs is an important factor for forming close spatial associations among ammonia and nitrite oxidizers (Johnston et al., 2019). However, the disintegration of activated sludge flocs in this study was observed during the cold season but no clear trend of the nitrifiers' washout with the treated discharge (TW-D and TW-W) was found. According to the obtained results, in TW-D and TW-W samples, the ammonia- and nitrite-oxidizing microorganisms reach up to 1.1% of the total community, while in the bioreactor they reach up to 15% (Saunders et al., 2013). Ammonia oxidizing bacteria (AOB) in TW-D and TW-W were represented mainly by the *Nitrosomonadaceae* family, genus *Nitrosomonas* (up to 0.3%), while the nitrite oxidizers (NOB) were mainly represented by *Nitrospiraceae* family, genus *Nitrospira* (up to 0.9%). Both genera were found be the dominant in many bioreactors (Limpiyakorn et al., 2006; Park and Noguera, 2004; Wang et al., 2012; Zhang et al., 2011). Note that *Nitrospira* is potentially able to completely oxidize ammonia to nitrate in the comammox process (Daims et al., 2015). It is worth noting that no ammonia-oxidizing archaea nor anammox bacteria were detected in samples collected from WWTP effluents.

In the case of denitrification, a wide variety of heterotrophic facultative anaerobes are capable of oxidizing organic compounds via nitrate respiration (Geets et al., 2007). In our study, potential denitrifying bacteria were represented by a wide range of *Proteobacteria* members, as well as some representatives from *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*. They ranged between 3.9.%–24.6% in TW-D, 4.3%–19.5% in TW-W and 3.0%–15.6% in environmental samples. The denitrification bacteria community in treated wastewater was much more diversified and represented by more taxa than in environmental samples. WWTP effluent was dominated by *Acinetobacter*, with some *Dokdonella*, *Dechloromonas*, and other taxa, present in the recipient (*Flavobacterium*, *Rhodobacter*, *Pseudomonas*, *Hyphomicrobium* and *Stenotrophomonas*). The presence of *Hyphomicrobium* among abundant denitrifiers in WWTPs was confirmed by Wang et al. (2014). On the contrary, *Thauera* and *Azoarcus*, reported by Wang as one of the main denitrifiers in tannery WWTP sludge, in this study were detected only in treated wastewater and only in minor shares ($\leq 0.01\%$), which suggest their strong sludge association. Recently attention is given also to the bacteria such as *Agrobacterium* sp., *Raoultella* sp., *Alcaligenes faecalis*, *Paracoccus versutus*, as well as *Pseudomonas stutzeri*, *Pseudomonas tolaasii*, and *Acinetobacter* sp., that are capable of using ammonium, nitrate or nitrite as a inorganic source of nitrogen and carry heterotrophic nitrification and aerobic denitrification. Among these genera, only *Pseudomonas* and *Acinetobacter* were detected in this study in higher shares (up to 0.5% and 2%, respectively) in treated wastewater samples.

In addition to nitrogen removal, both WWTPs perform enhanced biological phosphorus removal, which is carried out by polyphosphate accumulating organisms (PAOs) that can accumulate P in amounts exceeding their growth requirement. *Candidatus Accumulibacter* and *Tetrasphaera* are most frequently identified PAOs in full-scale wastewater plants even geographically distinct (Nielsen et al., 2019; Onnis-Hayden et al., 2020). Among other bacterial PAOs connected with activated sludge *Actinobacteria* (*Friedmaniella*, *Candidatus Microthrix*, *Microlunatus*, *Tessaracoccus*), *Proteobacteria* (*Dechloromonas*, *Pseudomonas*, Ca. *Accumulimonas*, *Quatrionicoccus*, *Malikia*, *Lampropedia*), and *Gemmatimonadetes* (*Gemmatimonas*) are also mentioned (Akbari et al., 2021). In this study, only *Candidatus Microthrix* (up to 23.31%), *Dechloromonas* (up to 0.6%), *Pseudomonas* (up to 0.5%) and *Gemmatimonas* (below 0.1%) were detected in TW-D and TW-W. Enhanced presence in treated wastewater of flocs forming bacteria, involved

in N/P removal may indicate the weakening of the sedimentation capacity of the activated sludge, as well as dissemination of such biomass via treated wastewater to receivers.

Another important aspect of WWTP effluent discharge into the environment is dissemination of pathogens or other emerging bacteria, because of correlation between recreational use of surface waters, and the occurrence of various infections (Pruss, 1998; Witzig et al., 2002). In this study, some potentially pathogenic genera were detected in the treated wastewater: *Mycobacterium* (up to 3.15%), *Bacteroides* (up to 1.51%), *Acinetobacter* (up to 1.94%), *Streptococcus* (up to 0.77%), *Arcobacter* (up to 0.5%) and *Pseudomonas* (up to 0.48%). Fecal indicators such as *Escherichia* and *Enterococcus* spp. were detected in most TW-D and TW-W samples and ranged between 0.01 and 0.11%, and up to 0.03%, respectively. No clear seasonal dependence was found, what was in line with cultivation-dependent analysis (see Section 3.2.2). In environmental samples these genera were observed sporadically, more frequently in winter than in summer samples, and in values not exceeding 0.01%. Also other bacteria from human gut microbial taxa (i.e. *Ruminococcus*) were found in all TW-D and TW-W samples (up to 0.8%), while in the recipient they were noted sporadically and in minor shares (<0.02%).

3.3.2. Microbial community of environmental samples

As already mentioned, the biodiversity of WWTPs effluents is rarely studied and not fully understood, however even less is known about the fate of wastewater-related bacteria in water reservoirs serving as WWTP effluent receivers. In this study, the microbial communities of environmental samples (MO-D, MO-W, VIS and GD) were highly similar on phylum level, irrespective of the presence or absence of wastewater discharge (Fig. 5c, e). These samples were dominated by eleven taxa, and similarly to treated wastewater, they showed a high percentage of *Actinobacteria* (classes *Acidimicrobiia* and *Actinobacteria*, together up to 41%), *Proteobacteria* (*Alpha–*, *Beta-* and *Gamma-* clades, together up to 57%) and *Bacteroidetes* (classes *Sphingobacteriia* and *Flavobacteriia*, together up to 12.5%). However, the most characteristic for the marine and estuarine samples were the high relative abundance of *Cyanobacteria* (21–49%), *Verrucomicrobia* (1–10%), and *Planctomycetes* (1–8%), which comprised 29–54% of the total microbial community, while in TW-D and TW-W these three phyla reached a maximum of 3.5%.

Most of the aforementioned bacteria (*Actinobacteria*, *Flavobacteriia*, *Sphingobacteria*, *Alpha-*, *Beta-* and *Gammaproteobacteria*) were also found by Berg et al. (2009) as accompanying cyanobacterial blooms. Interestingly, the several taxa dominating in environmental samples on genus level (constituting 48–75% of their total community) were also related to the bloom phenomenon. These genera included wide-spread marine clade *Pelagibacteraceae*, as well as *Synechococcus* and *Prochlorococcus* (small marine cyanobacteria) with *Flavobacterium* that contain strains capable of degrading cyanobacterial toxins (Berg et al., 2009), even if toxic *Nodularia* and *Anabaena* were not found. On the other hand, typically freshwater cyanobacterial genera were detected, e.g. *Dolichospermum* (up to 5%) or *Microcystis* (0.04%), however this is not surprising given the low salinity of the Baltic Sea waters and the inflow of riverine waters. Cyanobacterial blooms in the coastal zones are triggered by warmer temperatures and high nutrient availability, especially in areas impacted by treated wastewater (here: MO-D and MO-W) or by river discharge (here: VIS). This was confirmed by *Prochlorococcus* cyanobacteria showing higher abundance (2.6–8.6%) in summer samples (August/September), while did not exceed 1% in winter months (Nov and Feb). Nevertheless, HELCOM reports (Hansson and Öberg, 2012) reported that the surface blooms in 2012 were lower than average, compared with previous years. The NGS analysis also showed a relatively high percentage of sequences aligned to chloroplast or *Stramenopiles*, common marine single-cell eukaryotes (protists) that play a great role in the nutrient cycling in the oceans (Seeleuthner et al., 2018). Sequences identified as *Chloroplasts* covered even 19% of the microbial community in the environmental samples, while they did not exceed 3% in treated wastewater (with higher abundance in summer months, not exceeding 0.06% in winter). Despite their probable eukaryotic origin they were not excluded from the analysis, as they may correlate with the summer algal bloom or correspond to photosynthetic activity of some autotrophic bacteria.

Environmental samples on average presented lower abundance of nitrifying bacteria than treated wastewater. They were also represented by different taxa: Nitrospina (NOB) and Nitrosopumilus (AOA), which are ubiquitous in marine waters (Brown et al., 2013). They were more abundant in marine waters impacted by treated wastewater (MO-D and MO-W), where they reached up to 0.44%. In Vistula river estuary (VIS) they did not exceed 0.04%, while in Gdansk Deep 0.02%. Bacteria conducting anammox process were not detected, while ammonia-oxidizing archaea consisted up to 0.03%, and were represented by popular marine archaea *Nitrosopumilus*. In case of nitrite oxidizers, this group was represented mainly by the family *Nitrospiraceae*, genus *Nitrospina*, NOB, while *Nitrospira* was noted only in the Vistula river estuary (up to 0.01%). The denitrification community of environmental samples highly differed from treated wastewater. It was less numerous and dominated by two genera: *Flavobacterium* (phylum *Bacteroidetes*) and *Rhodobacter* (*Alphaproteobacteria*), only occasionally with the minor addition of other taxa, such as *Stenotrophomonas*, *Hyphomicrobium*, *Achromobacter*, *Pseudomonas* (mainly *Proteobacteria* representatives). It is worth noting that decaying blooms may also serve as an additional source of organic matter supporting the denitrification processes. Both nitrification and denitrification microorganisms were on average more abundant at the stations related to treated wastewater discharge (MO-D and MO-W) than on those not exposed to WWTP effluent.

3.4. Functional gene detection and quantification

As mentioned above, the N-cycle is transformed by a diverse microbial community, whose members are equipped in key genes. Thus, to fully recognize the nitrogen removal potential, the genes used as molecular markers of nitrification (amoA and nxrA) and denitrification (nirS, nirK, nosZ) were tested in this study together with 16S rRNA genes. It is worth to note that in environmental studies most of the available literature regarding gene abundance refers to soil or sediments rather than sea water.

Obtained data indicated that WWTPs effluent contained 16S rRNA target molecules ranging from 2.4×10^5 to 2.1×10^6 copies per μL of DNA (6.6×10^5 to 3.5×10^6 copies of 16S rDNA per 1 mL of the sample), while in environmental samples they varied in wider range from 4.2×10^4 to 1.4×10^6 copies per μL of DNA (1.6×10^4 to 2.5×10^6 copies of 16S rDNA per 1 mL of the sample), Fig. 7. These values, together with the abundance of N-functional genes, were compared with the data available in the literature (Fig. 8). In all samples of treated wastewater (TW-D, TW-W) the studied genes were present, with the highest abundance of nirS gene (encoding a haem-containing nitrite reductase) and nirK gene (encoding Cu-containing nitrite reductase), which are responsible for the reduction of nitrite to nitrogen oxide. Thus, both unrelated nir-genes, which never occur together in the same organism, are used as markers of denitrifiers. As expected, the nirS and nirK washed out from WWTPs with treated wastewater were noted on a similar level and ranged from 10^5 to 10^6 gene copies mL^{-1}, which was much lower than in activated sludge samples (Fig. 8c,d). In marine outfalls (MO-D and MO-W) and Vistula river estuary (VIS) both genes did not exceed 10^5 gc mL^{-1}, while in the Gulf of Gdansk (GD) they reached only 10^4 gc mL^{-1}.

The final step of denitrification, catalyzed by nitrous oxide reductase, was also analyzed by the presence of the nosZ gene, frequently used as a process biomarker (Fernández-Baca et al., 2018) and factor regulating the production of N_2O in different niches (Henry et al., 2006). In this study, the nosZ-based community was present in each analyzed sample, at the same order of magnitude from 10^4 to 10^5 gc mL^{-1}, except GD point, where they were less prevalent: 10^2 to 10^3 gc mL^{-1} (Fig. 7f).

Since NGS analysis has indicated that no AOA nor anammox bacteria were detected in samples collected from WWTP effluents (TW-W and TW-D), and AOA only occasionally appeared in marine outfalls (MO-D and MO-W) and in GD, it indirectly confirmed that organic matter stimulates

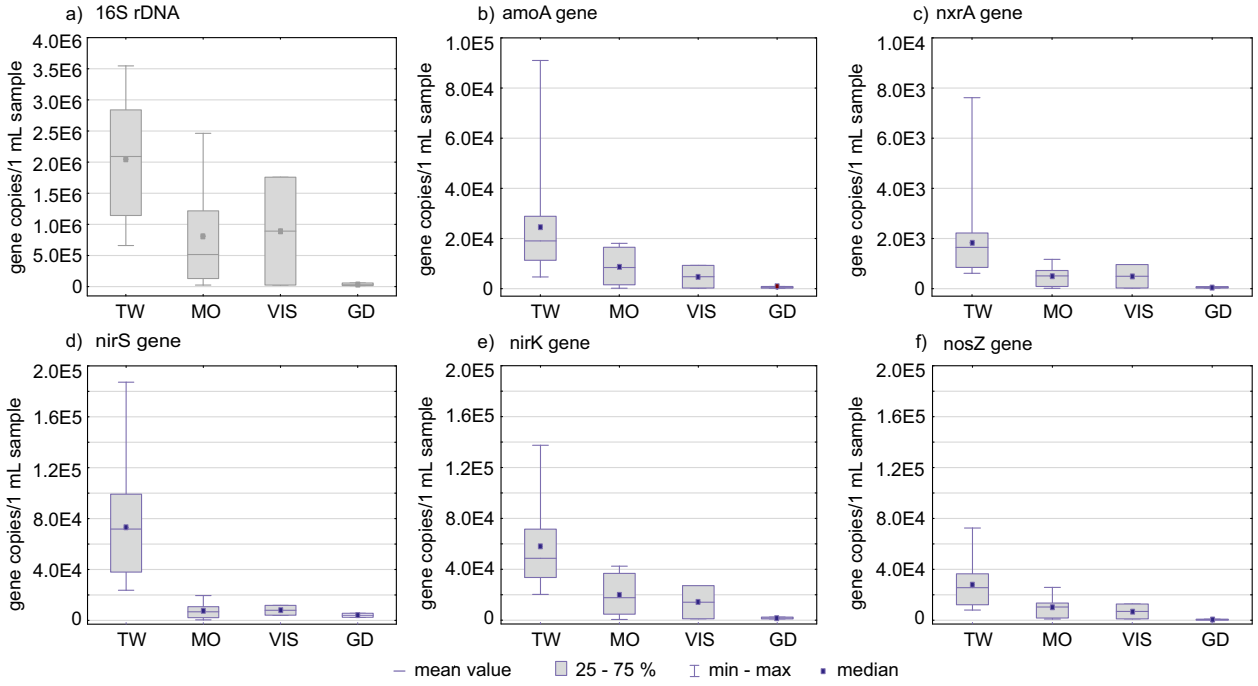

Fig. 7. Relative abundance of various DNA fragments and functional genes in different sample types (treated wastewater - TW, its marine outfall - MO, Vistula estuary - VIS, and Gdansk Deep - GD); a) 16S rDNA fragment, and functional genes involved in nitrification: b) amoA and c) nxrA, and denitrification: d) nirS, e) nirK and f) nosZ.

the denitrification and suppress anammox community, and that amoA-base community mainly consists of AOB. The amoA gene was detected in the same range as nosZ and linked mainly to *Nitrosomonadaceae* family. Gene nxrA, which is present in NOB and encodes the NO_2^- oxidation (Rani et al., 2017), occurred in lower quantity and could be linked mainly to family *Nitrospiraceae* (for details see Section 3.3.1).

4. Conclusions

Up to date, microbial community of the treated wastewater has still been rarely studied and is still largely unexplored area, especially in terms of its seasonal variability and the microbial influence on the receiving waters. In this study, the synergistic approach has been applied, combining chemical and microbiological analyses. Characteristics of the treated effluent from two major WWTPs in northern Poland were tested with a set of various cultivation-dependent and independent techniques.

The WWTPs' effluents showed some variations regarding the basic chemical parameters, however the mechanisms behind these changes and the link with microbial community composition fluctuations are not fully understood yet. Concentrations of the chemical parameters in the effluent seem to be more influenced by the season than the influent parameters. The results showed that not only the chemical quality of the effluents, but also their microbial community undergoes transformations throughout the year. Decreased wastewater treatment efficiency during winter was reflected in more numerous and smaller bacteria structured into small flocs in the treated wastewater. The most pronounced and unambiguous seasonal changes in the microbial community of the WWTP effluent can be seen in respect to temperature: in abundance of filamentous and bulking bacteria, and as a result of worse dispersed flocks sedimentation. Biomass washout appeared, however WWTP exploiters undertake measures to (e.g.: PIX/PAX dosing) to prevent this situation.

From the sanitary point of view, the abundance of fecal indicators in WWTP effluent did not present a clear seasonal pattern, neither it exceeded the current standard for bathing sites in the coastal waters impacted by treated wastewater. However, despite high treatment efficiency (<95%) in terms of chemical and microbiological parameters, the treated wastewater can still be a source of both nutrients and bacteria (also human-related ones) to the receiving waters. Treated wastewater discharge can also increase the biochemical potential of the receiving waters. The samples subjected to higher anthropogenic impact (MO-D, MO-W, VIS) consequently showed higher abundance of all the tested, potentially wastewater-related nitrification and denitrification bacteria, as well as N-cycling genes. To the best of the authors' knowledge, it is the first study that shows the presence of N-cycling genes in the treated wastewater and one of the few concerning their abundance in the marine water column.

CRediT authorship contribution statement

Conceptualization: AL, AK, KJ; Methodology: AL, KJ, AK; Software: MP, KJ, AK; Validation: AL, SFK, ARS; Formal analysis: MP, KJ, ARS, AK, AL; Investigation: AL, KJ, SFK, AK, ARS; Resources: AL, KJ, SFK, ARS; Data Curation: AL, MP, AK, Writing - Original Draft Preparation: AK, AL; Writing - Review & Editing: AK, AL, KJ; Visualization: KJ, MP, AK, Supervision: AL, SFK, KJ; Project administration: AL, Funding acquisition: AL, SFK, KJ.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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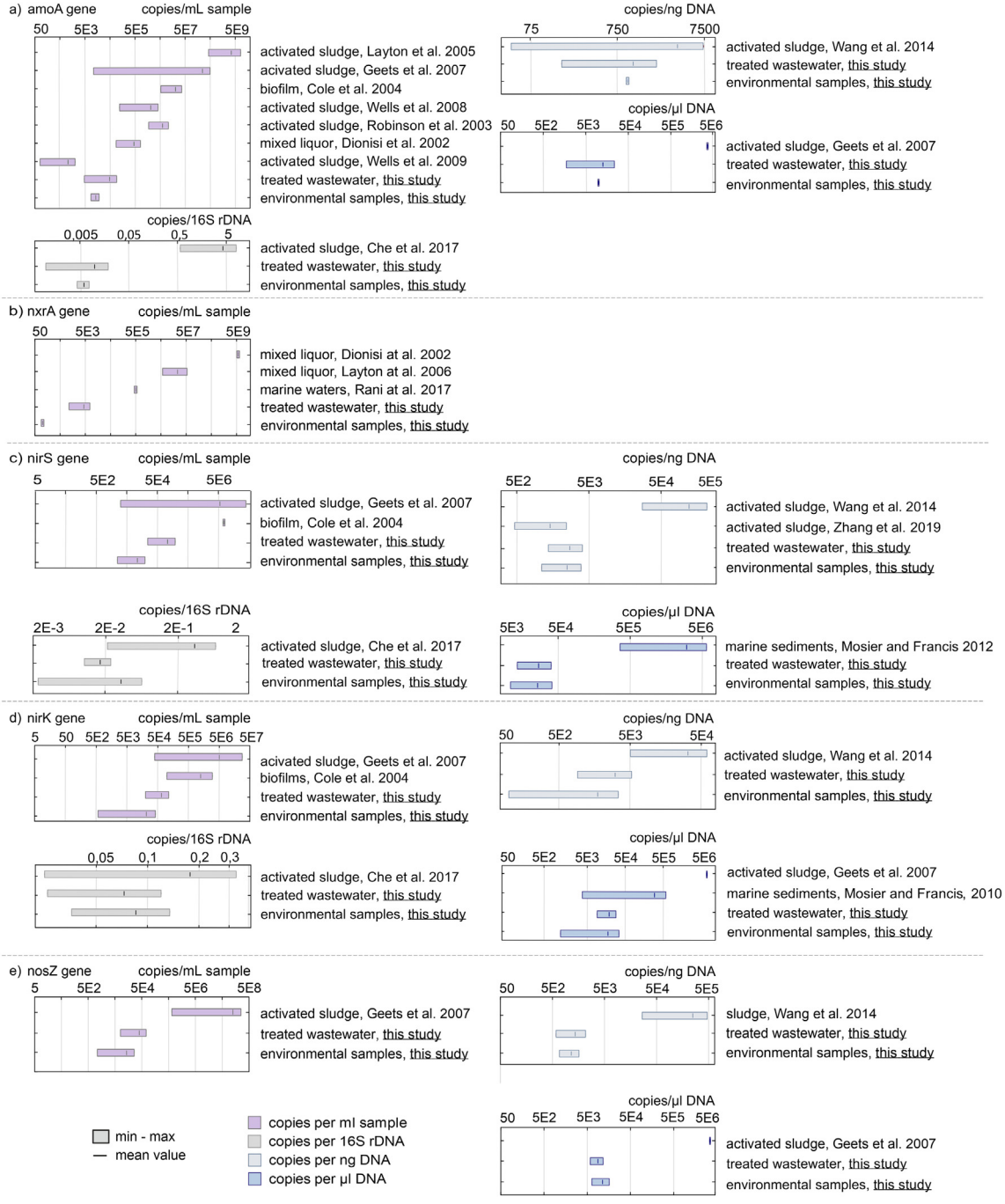

Fig. 8. Relative abundance of nitrification genes: a) amoA, b) nxrA, and denitrification genes: c) nirS, d) nirK and e) nosZ in treated wastewater and marine samples, compared with the literature data, expressed in various units (Che et al., 2017; Cole et al., 2004; Dionisi et al., 2002; Geets et al., 2007; Grüntzig et al., 2001; Kandeler et al., 2006; Layton et al., 2005; Rani et al., 2017; Robinson et al., 2003; Wang et al., 2014; Wells et al., 2009; Zhang et al., 2019).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154630.

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SUPPLEMENTARY MATERIALS - CLASSICAL MICROBIOLOGY AND METAGENOMIC APPROACH REVEAL THE MICROBIAL COMMUNITY AND BIOCHEMICAL POTENTIAL OF THE TREATED WASTEWATER AND ITS RECIPIENT

Table S1. Characteristics of the studied WWTPs: WWTP-W (Gdansk-Wschod) and WWTP-D (Gdynia-Debogorze). People Equivalent (PE) and average flow given as in 2015.

WWTP	Connected number of residents	PE [in BOD_5]	Designed capacity [m^3/d]	Average flow (min-max) [m^3/d]	Treatment technology			Influent Characteristic
					Mechanical	Biological	Chemical	
WWTP-W	570 000	742 500	120 000	92 958 (73 222-132 424)	screens, aerated grit chamber with a grease trap, and primary settling tanks	anaerobic/anoxic/oxic system (A2/O); advanced biological nutrient removal	PIX dosing system for occasional phosphorus removal	Industrial wastewater (11%) mostly from the food & chemical industry and shipyards. Hospital wastewater <1% of the total inflow; one infectious hospital effluent disinfected with UV
WWTP-D	360 000	476 000	73 000	55 294 (37 888-91 324)		Bardenpho system with simultaneous denitrification in Carroussel system; advanced biological nutrient removal		Industrial wastewater (10%) mostly from food, pharmaceutical & cosmetics industry, shipyards. Hospital wastewater (0,1%) discharged without disinfection

Table S2 Characteristic of the cultivation media used in the study

Medium	Symbol	Incubation temperature	Cultivated bacteria	Characteristic of enumerated bacterial colonies
Yeast agar	**AP**	22°C	psychrophilic	All growing on the medium
Yeast agar	**AM**	37°C	mesophilic	All growing on the medium
Coliform Agar	**C**	37°C	*Enterobacteriaceae*	All growing on the medium
			fecal coliforms	Blue to violet
Membrane Fecal Coliform Agar	**mFC**	44°C	*Escherichia coli*	Dark blue colonies

Table S3. Primer sequences used to amplify fragments from *nirS*, *nirK*, *nosZ*, *amoA* and *nxrA* genes in the denitrification pathway

Primer	Primer sequence (5'-3')	Reference
nirS1F	TACCACCCSGARCCGCGCGT	Kim et al. 2011
nirS 3r	GCCGCCGTCRTGVAGGAA	
nirK876	ATYGGCGGVCAYGGCGA	
nirK1040	GCCTCGATCAGRTTRTGGTT	
amoA-1-F	GGGGTTTCTACTGGTGGT	Li et al. 2012
amoA-2R	CCCCTCKGSAAAGCCTTCTTC	
nxrA-RT-F	GTGGTCATGCGCGTTGAGCA	Gerbl et al. 2014
nxrA-RT-R	TCGGGAGCGCCATCATCCAT	
nosZ-F	CGYTGTTCMTCGACAGCCAG	Throback et al. 2004
nosZ1622-R	CGSACCTTSTTGCCSTYGCG	

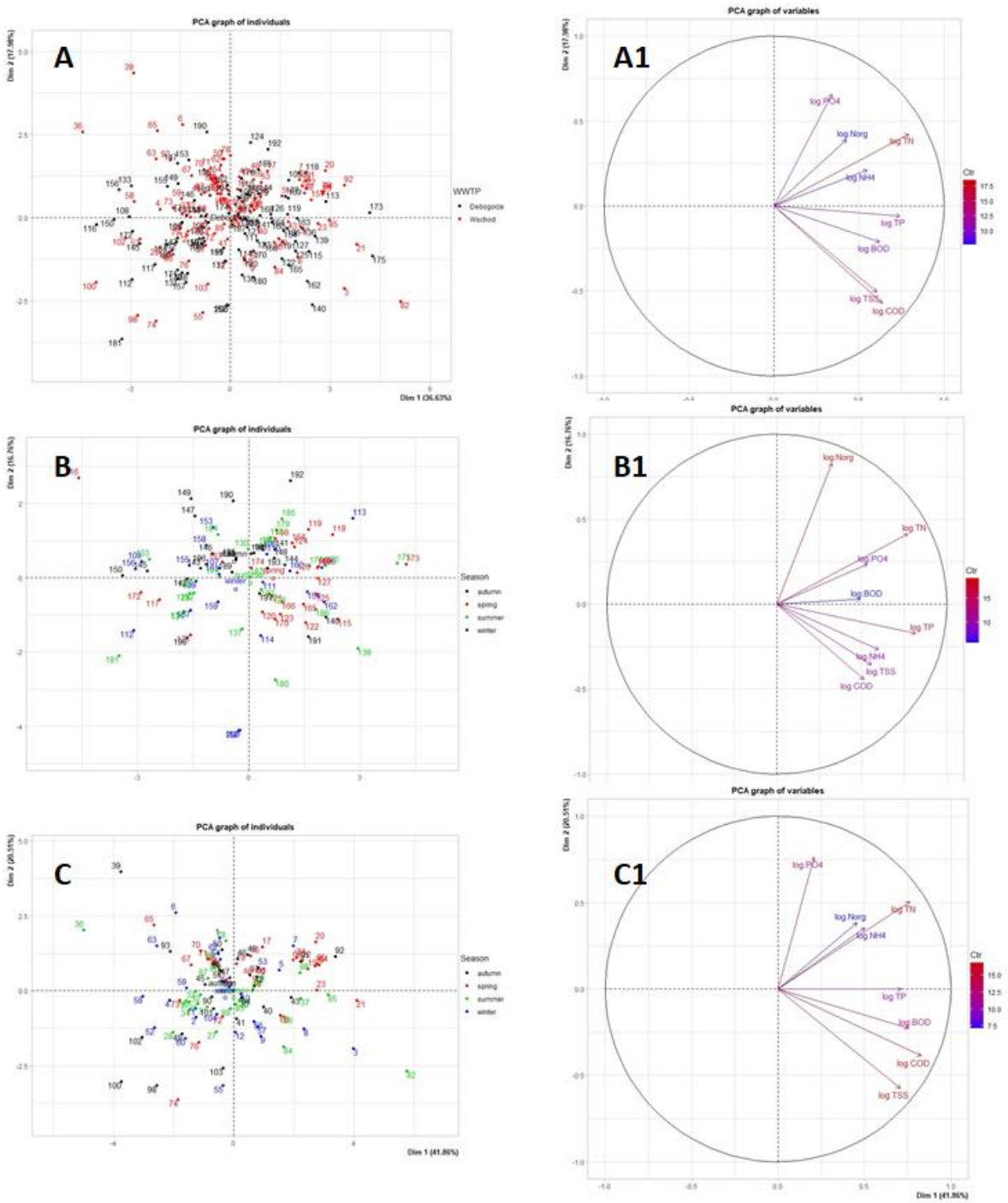

Figure S1. Principal Component Analysis (PCA) results for chemical parameters in raw wastewater of (A) both WWTPs, (B) WWTP-D and (C) WWTP-W. Numbers on the graph of individuals refer to the sample number. Additionally, the samples on Fig. B and C are coloured with respect to the season.

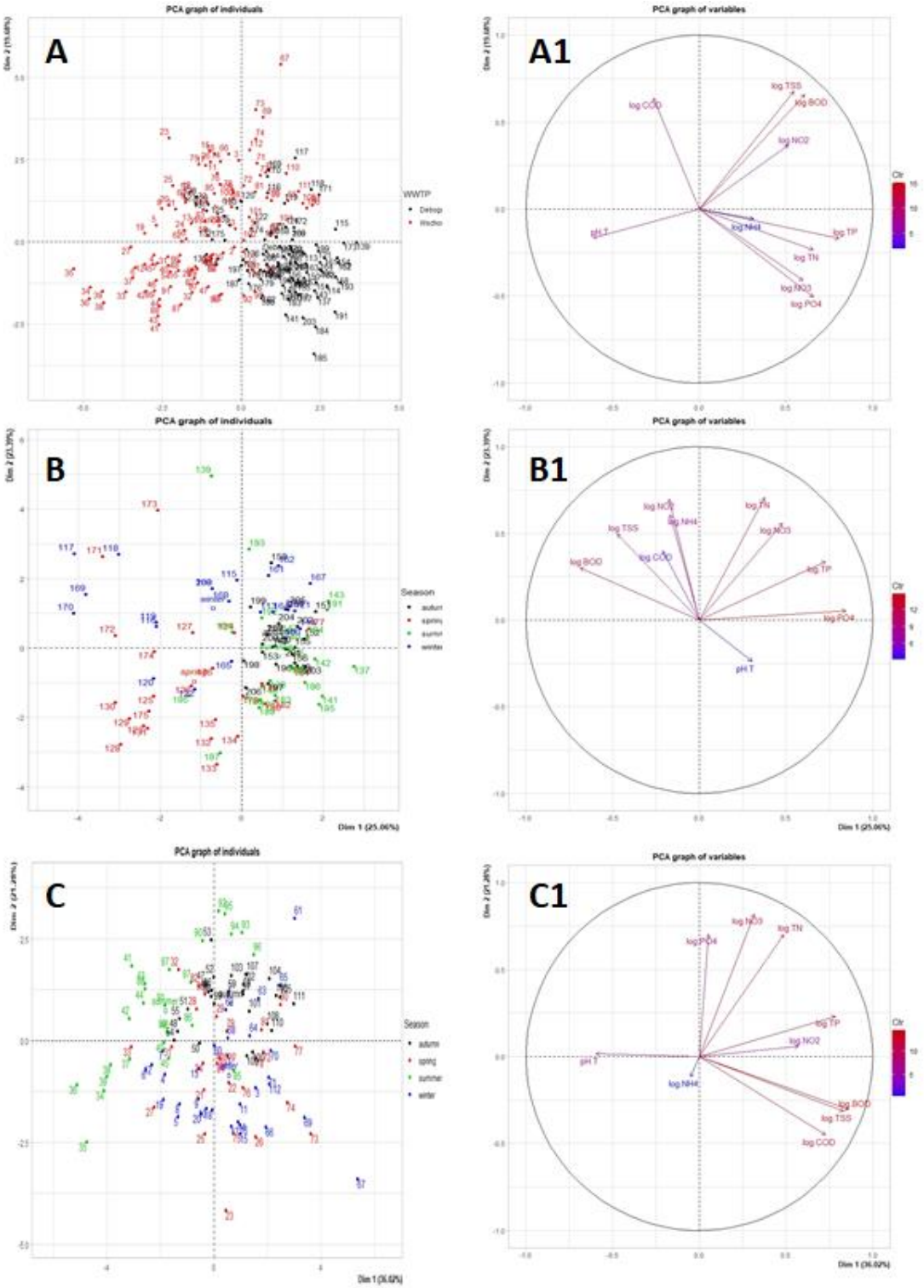

Figure S2. Principal Component Analysis results for chemical parameters in treated wastewater of (A) both WWTPs, (B) WWTP-D and (C) WWTP-W. Numbers on the graph of individuals refer to the sample number. Additionally, the samples on Fig. B and C are coloured with respect to the season.

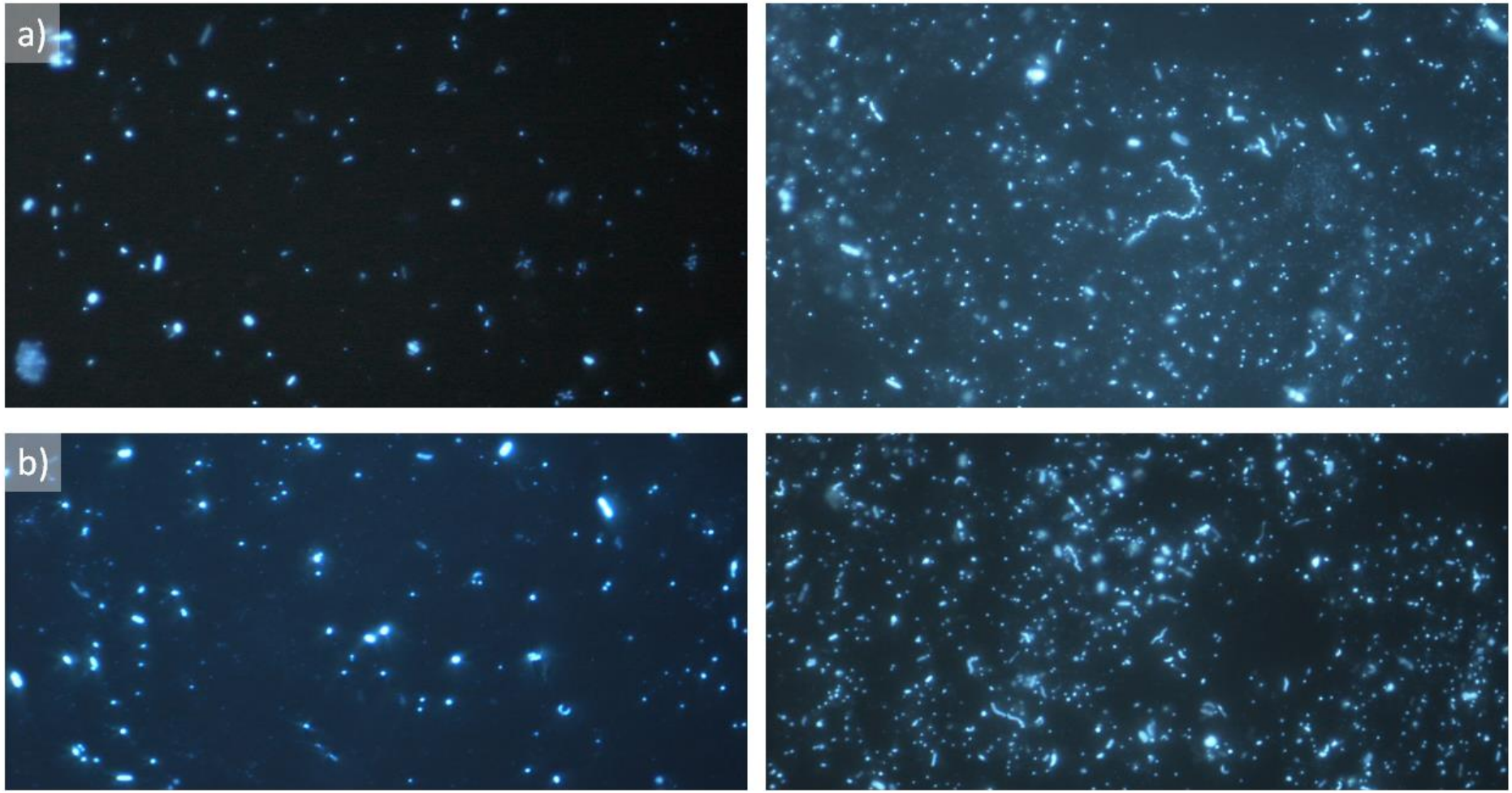

Figure S3. Photos of DAPI staining of treated wastewater from a) Gdynia-Debogorze WWTP and b) Gdańsk-Wschod WWTP. On the left samples from June, on the right from December

Manuscript IV

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A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants

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ABSTRACT

The World Health Organization Global Action Plan recommends integrated surveillance programs as crucial strategies for monitoring antibiotic resistance. Although several national surveillance programs are in place for clinical and veterinary settings, no such schemes exist for monitoring antibiotic-resistant bacteria in the environment. In this transnational study, we developed, validated, and tested a low-cost surveillance and easy to implement approach to evaluate antibiotic resistance in wastewater treatment plants (WWTPs) by targeting cefotaxime-resistant (CTX-R) coliforms as indicators. The rationale for this approach was: i) coliform quantification methods are internationally accepted as indicators of fecal contamination in recreational waters and are therefore routinely applied in analytical labs; ii) CTX-R coliforms are clinically relevant, associated with extended-spectrum β-lactamases (ESBLs), and are rare in pristine environments. We analyzed 57 WWTPs in 22 countries across Europe, Asia, Africa, Australia, and North America. CTX-R coliforms were ubiquitous in raw sewage and their relative abundance varied significantly (< 0.1% to 38.3%), being positively correlated ($p < 0.001$) with regional atmospheric temperatures. Although most WWTPs removed large proportions of CTX-R coliforms, loads over 10^3 colony-forming units per mL were occasionally observed in final effluents. We demonstrate that CTX-R coliform monitoring is a feasible and affordable approach to assess wastewater antibiotic resistance status.

1. Introduction

Over the past few decades, the global spread of antibiotic resistance has increased to alarming levels, approaching what has been coined as the "post-antibiotic era" (Heymann et al., 2007). While this phenomenon is traditionally linked to healthcare-associated infections (Ventola, 2015), it is believed that animal husbandry, aquaculture facilities and urban wastewater treatment plants (WWTPs) also contribute to propagating antibiotic resistance by discharging antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), and residual concentrations of antibiotic compounds into downstream aquatic and terrestrial environments (Berendonk et al., 2015; Tripathi and Cytryn, 2017). Standard microbial water quality assessment methods, including quantification of intestinal coliforms and enterococci, are frequently applied for monitoring microbial quality in wastewater treatment effluents (Tallon et al., 2005). While the methods based on these indicators are considered to be reliable for the detection of fecal contamination, they do not assess antibiotic resistance levels, a new threat that increasigly needs routine evaluation. When associated with mobile genetic elements (MGEs), ARGs can be horizontally transferred between bacterial cells, even across distinct lineages, thereby facilitating antibiotic resistance dissemination (Smets and Barkay, 2005). Consequently, ARGs have been described as "contaminants of emerging concern" (Pei et al., 2006; Pruden et al., 2013) and have motivated a plethora of studies targeting ARGs in WWTPs effluents and downstream environments (Marano et al., 2019; Narciso-da-Rocha et al., 2018; Rizzo et al., 2013; Wang et al., 2014). Most of these studies rely on molecular biology methods, which provide highly informative data on the ARGs present in these environments. These studies facilitated efforts to standardize analytical methods for measuring selected ARGs in WWTP effluents and receiving water bodies (Rocha et al., 2018). However, the application of molecular methods also requires specialized equipment, expensive reagents and proficient technical staff, making them less suitable for routine, widespread WWTP monitoring (Bürgmann et al., 2018; Manaia et al., 2018). Moreover, these methods do not interrelate with conventional (culture-based), globally standardized microbiological water quality indicators. Although culture-based methods often overlook a large fraction of the wastewater microbiota, they can provide fundamental data on antibiotic resistance trends of individual species and strains coupling antibiotic resistance phenotypes to individual bacterial isolates of clinical concern. A major bias of culture-based methods comes from omitting unknown and often strictly

environmental bacteria that are profuse in wastewater samples but do not grow on standard culture media, as suggested by Bengtsson-Palme et al. (2016). The use of culturable coliforms indicators, such as *Escherichia coli*, has been globally adopted due to its low-cost and ease of implementation for assessing fecal contamination; however, previous experience has shown that antibiotic-resistant bacteria of human/animal origin thriving in wastewater are not only fecal coliforms but include a wider range of enterobacteria (Ferreira da Silva et al., 2007; Vaz-Moreira et al., 2015). Therefore, a surveillance system that could tackle these aspects was designed and tested as presented in this paper. Targeting antibiotic-resistant coliforms may be especially relevant from human health perspective given the realization that horizontal transfer of MGEs occurs much more frequently between phylogenetically closely related organisms and that this process is especially prevalent among coliforms (Popa and Dagan, 2011; Vaz-Moreira et al., 2015).

Within the framework of the European Union's COST Action ES1403: *New and emerging challenges and opportunities in wastewater reuse* (NEREUS), we conceived a simple experimental scheme for estimating the abundance of antibiotic resistance using standard methods that can easily be applied and interpreted by stakeholders like WWTP operators. The method makes use of standard membrane fecal coliform agar (mFC Agar) medium to quantify coliforms (Rompré et al., 2002), but in tandem also quantifies antibiotic-resistant coliforms by amending the mFC Agar medium with cefotaxime, a third-generation cephalosporin, at a clinically-relevant concentration (Heil and Johnson, 2016). Resistance to this antibiotic has considerable clinical significance due to the increasing abundance of resistant bacteria-harboring extended-spectrum β–lactamase (ESBL) genes, which are frequently associated with multidrug resistance and widespread in the environment (Bradford, 2001; Pitout and Laupland, 2008). This is especially true for clinically relevant "priority pathogens" from the *Enterobacteriaceae* family including *Escherichia coli*, *Klebsiella pneumoniae,* and *Enterobacter* spp., but is also pertinent to other Gram-negative "ESKAPE" pathogens such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Boucher et al., 2009).

We evaluated the applicability and the potential epidemiological indicative capacity of this method by launching a global initiative that encompassed WWTPs from five continents (Fig. 1) in two independent sampling campaigns. For most of the analyzed WWTPs, the method was applied to test raw (influent) and treated (effluent) sewage. Stakeholders provided metadata related to specific treatment plant parameters, further used to test correlations between specific factors and antibiotic resistance levels. The aims of this study were (1) to quantify the absolute and relative abundance of cefotaxime-resistant (CTX-R) coliforms in raw sewage (influent) in different locations and to investigate potential predictors of their estimated abundances; (2) to estimate the ability of WWTPs to remove CTX-R coliforms from wastewater, and (3) to quantify the load of CTX-R coliforms discharged (effluent) from WWTPs to downstream aquatic and terrestrial environments.

2. Materials and methods

2.1. Site description and WWTP characteristics

A total of 57 WWTPs from 22 countries were sampled. Most (47 of 57) of the analyzed WWTPs were located in Europe, one was located in Africa, six were in Asia, one in Australia and two WWTPs were in North America. In 14 of the 22 participating countries, two or more WWTPs were sampled (Supplementary Table 1).

For each WWTP, grab samples were taken in two campaigns. The first campaign consisted of three sampling dates, once per month, between December 2016 and February 2017; for the second campaign, two sampling dates were chosen between May and October 2017 (Supplementary Table 1). Participating groups were provided with detailed protocols for sampling, sample processing and cultivation and bacterial enumeration and completed a questionnaire on metadata related to the country and the WWTPs. All experimental data were

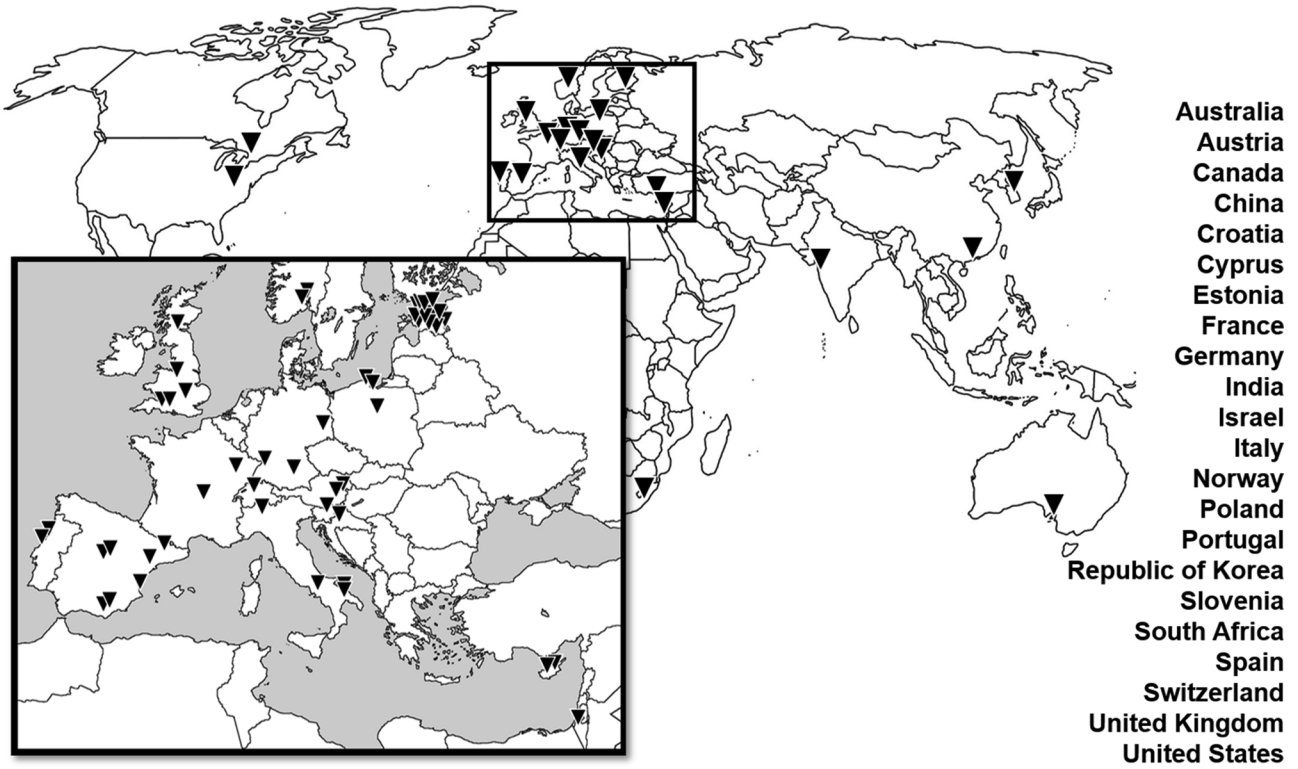

Fig. 1. A geographical overview of the global survey on cefotaxime-resistant coliforms. Black triangles show geographic locations of the participating countries where WWTP samples were collected. Square insert shows targeted European WWTPs.

screened, quality checked, and assembled as described below before performing statistical analyses.

2.2. Metadata collection

The participating groups filled out a questionnaire with data related to WWTP catchment population size and influent characteristics, the WWTPs' technical setup and operational conditions, physicochemical characteristics of the final effluent, meteorological conditions of WWTP sites, antibiotic consumption in each country and the WWTPs' GPS coordinates. Meteorological conditions were retrieved from official national forecast online web databases, using average monthly temperatures of sampling days. Antibiotic consumption, available for European countries only, was retrieved from the antimicrobial use database of the European Centre for Disease Prevention and Control (www.ecdc.europa.eu) using the filters: "2016", "β-lactam antibacterials and penicillins", and "primary care sectors". Antibiotic consumption is indicated as 'defined daily doses (DDD) per 1000 inhabitants per day'.

2.3. Sampling

Grab samples were collected during weekdays and processed on the day of sampling as described below. Raw sewage (influent) after primary sedimentation and effluents after secondary biological treatment and/or after disinfection were sampled. From a total of 57 WWTPs evaluated in this study 54 applied conventional activated sludge (CAS) with secondary sedimentation, two WWTPs used membrane bioreactor (MBR), and one used trickling filter (TF) treatment technologies (Supplementary Table 1). Distinct disinfection processes were applied in 22 WWTPs, using chlorination (n = 7), UV radiation (n = 11), chlorination and UV radiation (n = 1), membrane filtration (n = 2) or ozonation (n = 1). Three WWTP treatments applied the disinfection process seasonally, two used UV radiation in the summer and one chlorination in the winter (Supplementary Table 1).

2.4. Enumeration of bacteria

Membrane fecal coliform Agar (mFC, Difco® with 0.01% (w/v) Rosolic Acid (Difco®)) was prepared according to manufacturer instructions. For the quantification of CTX-R coliforms, mFC Agar was supplemented with cefotaxime sodium salt (Sigma®) at a final concentration of 4 μg mL^{-1}. This concentration was based on the minimal inhibitory concentration (MIC) breakpoint levels for *Enterobacteriaceae,* documented by CLSI (Clinical and Laboratory Standards Institute, Wayne, PA, 2010), which is more stringent than the EUCAST MIC breakpoints of > 2 μg mL^{-1} (European Committee of Antibiotic Susceptibility Testing http://www.eucast.org/clinical_breakpoints/) and would also be more selective against false positives and intermediately resistant colonies. Culture media were prepared no more than three days before sampling and dispensed in 60 mm diameter Petri plates. When the liquid culture medium reached 55 °C, the approporate volume of the fresh concentrated filter-sterilized antibiotic stock solution was added before pouring plates. Samples were serially diluted and 1 mL from 2 to 4 consecutive 10-fold serial dilutions were filtered through 47 mm diameter 0.45 μm pore size nitrocellulose sterile membrane filters on mFC Agar plates (in triplicate) with and without cefotaxime. For less turbid water samples (*i.e.*, effluents) up to 100 mL were filtered. Cultures were incubated for 24 h at 37 °C. Incubation time was not prolonged beyond 24 h to avoid biases associated with the inactivation of cefotaxime in the culture medium by the CTX-R bacteria that produce extracellular cefotaxime degrading β-lactamases. Moreover, even though the mFC Difco® protocol recommends incubation at 44 °C, we specifically chose 37 °C (i) to reduce curing of plasmids harboring ARGs, which has been shown to occur at elevated temperatures (Trevors, 1986), and (ii) to avoid possible functional thermal instability previously reported for CTX-M enzymes and other β-lactamases at higher temperatures (He et al., 2016; Vanhove et al., 1995). To validate our choice of incubation temperature we compared the identity of the bacterial groups recovered under both the conditions and criterion used (*i.e.*, incubation at 37 °C vs. 44 °C), (see Section 2.7). Under these conditions, the blue colonies enumerated on mFC Agar with or without cefotaxime were considered as presumptive coliforms. The limit of detection (LOD) of the method was calculated as the minimal number of colonies that could be enumerated in the highest volume analyzed. Extrapolation of the generated results suggests that the LOD was approximately 0.3 colony forming units (CFUs) mL^{-1} (with 1 being the minimum number of observable colonies on triplicate mFC plates for 1 mL of filtered water); however, it should be noted that in a few cases filtered effluent volume was increased to 10 or 100 mL when water samples were particularly clear. To improve reproducibility, it was recommended that each step in the procedure (media preparation, dilutions, filtration and colony enumeration) be conducted by the same person processing the samples of a given WWTP. Participating groups were provided with a standard spreadsheet to collect the results of bacterial enumeration and were asked to report the date and location of sampling.

2.5. Quality control criteria

For CFU enumeration, the following criteria were applied by all participating groups: (i) optimally, only filtering membranes with 10–80 CFUs were included in analyses; (ii) when this range of CFUs was not available, the highest dilution with a countable number of CFU was used; (iii) when a selected dilution had replicates with inconsistent CFU counts (defined as a standard deviation (SD) > 20% of the mean), a different dilution range was chosen according to the above inclusion criteria; and (iv) whenever technical or methodological issues from WWTPs or during sample preparation were reported, data were discarded regardless of the above inclusion criteria.

After the initial data trimming and quality control, data from 228 influent samples, 199 secondary effluents (MBR or CAS final effluent or prior to disinfection) and 79 effluents after a disinfection step were collected (Supplementary Table 1).

The relative abundance of CTX-R coliforms was calculated by dividing the number of CFUs mL^{-1} on mFC Agar supplemented with cefotaxime by the number of CFUs mL^{-1} on mFC agar without antibiotic for each of the three replicates. The WWTP removal efficacy for coliforms and CTX-R coliforms was calculated as either percent or log-unit removal (respectively) using the following equations:

$$\frac{CFUsinfluent - CFUsoutflow}{CFUsinfluent} \times 100;\ \log\left(\frac{CFUsinfluent}{CFUsoutflow}\right)$$

2.6. Statistical analyses

To evaluate whether coliform abundance in influents and effluents would be a predictor for CTX-R coliform levels we used F-tests to compare variances. Predictor factors for resistance in influents were also investigated in linear regression analyses comparing coliforms and CTX-R coliforms, to local geographical areas of WWTPs and to the described β-lactam antibiotic consumption indicator (2.2). Non-parametric Mann Whitney U-tests were used to compare coliforms and CTX-R coliforms CFU mL^{-1} in influents at selected regional atmospheric temperature ranks. Removal efficacy of coliforms and CTX-R coliforms from influents after CAS process (*i.e.* secondary effluents) from WWTPs located in regions with different temperatures were compared with non-parametric Mann Whitney *U* test. Statistical analyses were conducted using GraphPad Prism® version 6.00 for Windows (GraphPad Software, La Jolla, CA, United States) and were interpreted using a significance level set at $p \leq 0.05$.

2.7. Methodological validation through bacterial characterization

In parallel with the surveillance study, the diversity of bacteria targeted by the procedure used was assessed and the taxonomy of selected bacteria was determined in a parallel series of tests, with the following objectives in mind: (i) assess the taxonomic composition of wastewater derived bacteria forming blue colonies on mFC Agar at 37 °C and compare with that of blue colonies recovered at 44 °C (the recommended incubation temperature for isolating fecal coliforms on mFC Agar); (ii) assess if, as hypothesized, bacteria with acquired antibiotic resistance are more prevalent when incubated at 37 °C than at 44 °C.

These bacterial characterization experiments involved eight partners of different geographic locations (USA, Australia, Israel, Portugal, UK, Germany, Croatia, Poland). Samples from secondary effluents (only) were processed as described above and incubated at 37 °C and 44 °C. From plates incubated at 37 °C or 44° (on agar with and without cefotaxime) a total of 30 blue CFUs were selected, isolated, and taxonomically characterized using either 16S rRNA gene sequence anaylsis or MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectronomy; detailed protocols for both are described in the Supplementary Material). To elucidate the potential impact of incubation temperature on the relative abundance of CTX-R isolates, the recovery capacity of CTX-R *E. coli* isolates was evaluated at the two compared incubation temperatures; only *E. coli* were investigated due to their clinical relevance and the fact that they were the most abundant species identified, suitable for statistical investigation. The amount of total and CTX-R *E. coli* CFU per mL in raw samples was estimated, for each assembly of 30 isolates, from each group, from the four tested conditions using the formula below:

$$\left(\frac{\text{N identified E. coli}}{\text{N total identified}}\right) \times (\text{N blue CFUs on filter} \times \text{Df})$$

where the first factor of the multiplication is the proportion of validated *E. coli* out of the 30 isolates from each condition, and the second factor is the total number of blue CFUs per mL in the raw sample (blue CFUs on filter multiplied by the dilution factor, Df). Thereafter, the ratios between the estimated *E. coli* CFU per mL retrieved at 37 °C and 44 °C were calculated for mFC Agar and mFC Agar + CTX (respectively).

3. Results and discussion

Numerous studies have explored antibiotic resistance in WWTP influents and effluents using both traditional cultivation techniques (Hoelle et al., 2019) and culture-independent molecular analyses that target ARGs (Petrovich et al., 2018). Data generated from these studies is very important, but because they are generally constrained to individual facilities or local regions, they lack perspective on the global dimensions of antibiotic resistance in WWTPs. In contrast, two recent international collaborative initiatives have facilitated comprehensive perceptions of antibiotic resistance in WWTPs. By applying shotgun metagenomic analysis of untreated sewage in 79 sites from 60 countries around the world, Hendriksen et al. (2019) found systematic geographical differences in abundance and diversity of ARGs, with little correlation to antibiotic use or bacterial taxonomy. While specific factors were difficult to identify, they concluded that socioeconomic factors such as poor sanitation were primary drivers of ARG propagation. A recent pan-European study conducted by Pärnänen et al. (2019) that targeted over 250 ARGs and associated MGEs in influents and effluents of 12 WWTPs from seven countries using highly-parallel quantitative PCR found a north–south distribution of ARGs, consistent with trends observed for clinical isolates (ECDC, 2018). While both of these studies significantly contribute to our understanding of antibiotic resistance in WWTPs, they are based on methodologies that are extremely costly and require (i) advanced expertise in manipulation and data analyses, (ii) DNA/RNA extraction kits, (iii) expensive equipment (e.g. real-time PCR, sequencing facilities) and (iv) Physical Containment facilities (*i.e.* PC2, PC3), all normally not available in WWTP labs. We therefore focused on implementing a simple culture-based approach that can be routinely applied or analyzed on a global scale for elucidating the abundance of clinically-relevant antibiotic-resistant bacteria in WWTPs. Such a method would only require a simple modification of already existing microbial analytical procedures at WWTPs compared to the costly alternative methods described above (i.e qPCR and metagenomics). A comparative cost analysis to better elucidate on such expenses is given in Supplementary Table 5. Of note, the described method should not be intended as an alternative to the use of mFC Agar® plates incubated at 44 °C, as the populations targeted by the two conditions (*i.e.* 44 °C and 37 °C) are slightly different as elaborated below).

Comparison of the two incubation temperatures described in section 2.7 revealed that mFC Agar cultures (with/without cefotaxime) incubated at 44 °C selected for members of the *Enterobacteriaceae* family, with the majority of these being *E. coli* (> 71% of the identified), as expected. *E. coli* was also the most abundant species detected from the mFC screened cultures incubated at 37 °C, although at a lower frequency (> 31% of the identified). Other genera of the family *Enterobacteriaceae* were detected on plates incubated at 37 °C with or without cefotaxime, and these included *Citrobacter, Enterobacter,* and *Klebsiella* spp., all genera that include opportunistic human pathogens

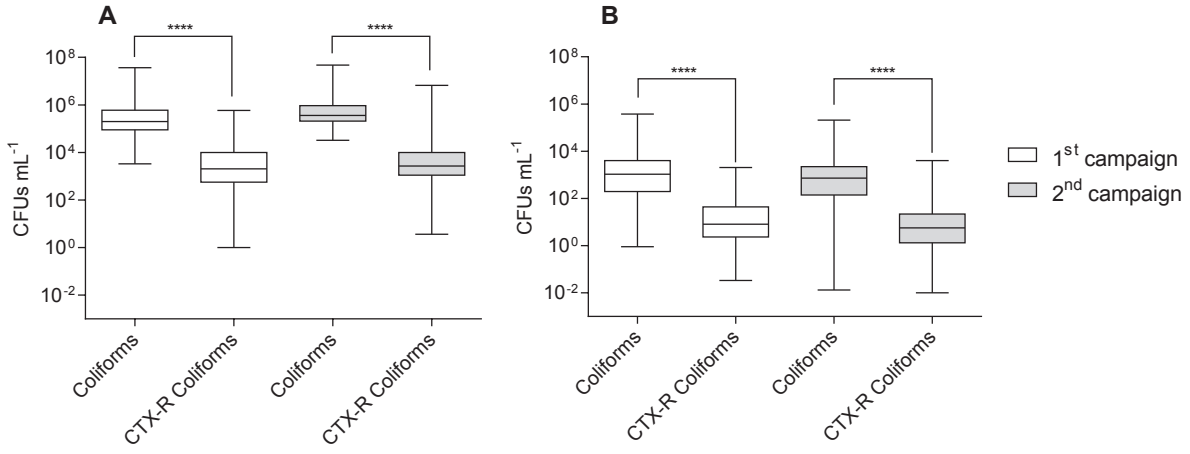

Fig. 2. Distribution of coliforms and CTX-R coliforms in 57 sampled WWTPs. A: influents, based on 228 samples (146 from the 1st campaign, and 82 from the 2nd). **B**: effluents, on 244 samples (150 from the first campaign and 94 from the second). Asterisks refer to a p-value < 0.0001 on an F–test to compare the variance between samples. Box plots show means and quartiles, whiskers indicate the minimum and maximum values.

(Supplementary Fig. 1). Non–*Enterobacteriaceae* were also detected in cultures incubated at 37 °C, mainly *Aeromonas* spp. (*A. caviae* and *A. hydrophila*), representing < 23% of all identified isolates, while other genera and species represented < 3% (Supplementary Fig. 2). Noticeably, although the amount of *E. coli* among the blue colonies counted on mFC Agar incubated at 37 °C was lower than those counted on mFC Agar incubated at 44 °C, both estimates were within the same order of magnitude when considered as CFU per mL (average ratio of 6 comparisons between the two incubation temperature = 1.1 ± 0.4; Supplementary Fig. 3). On the contrary, incubation of mFC Agar plates supplemented with cefotaxime at 37 °C facilitated higher ($p < 0.05$) recovery of CTX–R *E. coli* than the same medium incubated at 44 °C (by a factor 2.8 ± 1.5 across 6 comparisons), supporting the hypothesis that acquired antibiotic resistance is more stable at 37 °C than at 44 °C. These findings suggest that despite the slightly elevated number of false positives (non coliforms and aeromonads) generated, the significantly higher level of CTX–R *E. coli* detected at 37 °C supports its application in targeting the resistant *E. coli* fraction of wastewater. At least for the tested fraction of the coliform community, these findings support the hypothesis that CTX–R coliforms might be selected against or lose resistance determinants such as plasmids at higher temperatures, biasing the evaluation of their proportion in the original sample (Trevors, 1986).

3.1. Abundance of CTX-R coliforms in raw sewage

It can be assumed that CTX-R coliforms in influents primarily originate from fecal matter in the sewage entering the individual WWTP. Fig. 2A and Supplementary Table 2 summarize the abundance of CTX-R coliforms in influents observed in the two global survey campaigns. For both the sampling campaigns, between WWTPs, the variance of CTX-R coliforms was significantly higher than that of total coliforms (Fig. 2A). This variance was also reflected in the relative abundance of CTX–R coliforms (as percentage) in the various WWTPs, which ranged between < 0.1% and 38.3%, with a global mean of 2.7%. These observations highlight both, the ubiquity of CTX-R coliforms in untreated wastewater sewage and the highly unequal geographical distribution of this resistance phenotype.

Strong variation in CTX-R coliforms levels between the different WWTP influents may stem from a myriad of factors such as antibiotic use, sanitary conditions, and various abiotic factors. Recent studies have suggested significant correlations between climate (aerial temperature) and antibiotic resistance. MacFadden et al. (2018) performed a *meta*-analysis of antibiotic-resistant *E. coli*, *K. pneumoniae* and *Staphylococcus aureus* data from different geographical regions of the US (1.6 million strains, from 223 facilities across 41 states between 2013 and 2015). They found that regional temperature was the most significant factor associated with antibiotic resistance, where a 10 °C increase in minimum temperature coincided with approximately 2–6% increase in resistance in the three targeted pathogens. Temperature is very often underestimated in wastewater microbial ecology, in contrast to freshwater microbial ecology, where it is considered one of the most significant parameters in determining the bacterial community composition of rivers and lakes (Likens, 2010). To investigate the potential correlation between local temperature (monthly temperature, at the collection site, of the day of sampling based on meteorological data) and the abundance of coliforms and CTX-R coliforms in raw sewage, 228 influent samples comparing monthly mean temperatures with an absolute abundance of coliforms and CTX-R coliforms were analyzed. The rationale for addressing ambient temperature stemmed from three factors: (i) ambient temperature can significantly influence sewage temperature within activated sludge where aeration is applied (due to differences in air temperature); (ii) temperature can have a major impact in combined sewer systems that receive rain/melted snow; (iii) temperature is often a surrogate parameter for geographical latitude as north–south gradients, which are frequently reported in clinical ABR monitoring studies (MacFadden et al., 2018; Pärnänen et al., 2019). We observed a slightly positive correlation between $\log_{10}$ converted CFU values and temperature, which was more prominent below 15 °C for both coliforms ($R^2 = 0.22$) and CTX-R coliforms ($R^2 = 0.1$) (Fig. 3, Supplementary Figure 9). Upon the observed trend inversion of the dataset above and below this indicated temperature, we compared the CFU abundance in samples from places with monthly regional temperatures below 5 °C and above 15 °C (as monthly averages), represented by two groups with a similar number of samples (77 samples < 5 °C and 72 samples > 15 °C) for both coliforms and CTX-R coliforms at both temperature ranges (Fig. 4). A version of Fig. 4 of the complete datapoint split into three ranks is presented in Supplementary Figure 10.

Both, total and CTX-R coliforms (CFUs mL^{-1}) were generally more abundant in WWTPs influents from areas with higher temperatures (Fig. 4) and this observation was not dependent on specific locations or sampling campaign, as data from the WWTPs were evenly distributed across the two temperature ranks. However, no clear statistical correlation with temperature was observed when looking at the relative abundance of CTX-R coliforms (Supplementary Fig. 4) suggesting that other confounding variables might have influenced the observed relative abundance in sewage. Despite the fact that correlation does not necessarily entail causation, it was speculated that a higher abundance of coliforms in WWTPs operating in geographical areas with temperatures above the 15 °C threshold may be explained by a multitude of climatic and potential indirect socioeconomic factors that were not taken into consideration in this study. The difficulty in disentangling temperature from other factors such as antibiotic use, population

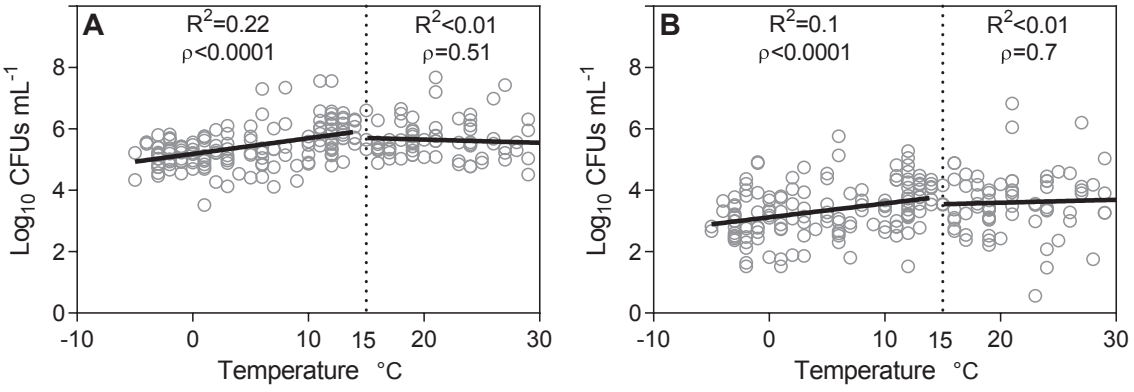

Fig. 3. Linear regression of $\log_{10}$ converted coliforms (A) and CTX-R coliforms (B) in WWTP influents as a function of monthly average temperature in the sampled areas. Datasets are split into temperatures ranks below and above 15 °C (highlighted by dotted lines); R^2 and p-values from linear regression are reported for each tested interval. A version of these data analyses without splitting of temperature intervals is given in Supplementary Figure 9.

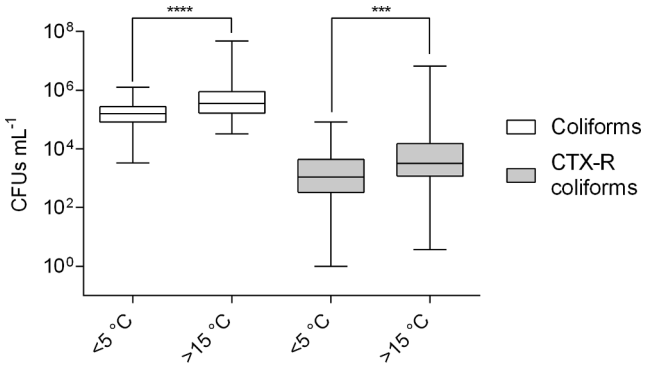

Fig. 4. Comparison of coliforms and CTX-R coliforms abundance in WWTPs' influent sewage in selected temperature ranks. Asterisks refer to a Mann-Whitney *U* test with given p-values < 0.0001 (****) and p < 0.001 (***). Sample size coliforms: < 5 °C n = 77, > 15 °C n = 72; sample size CTX-R coliforms < 5 °C n = 76, > 15 °C n = 72. Box plots show percentiles together with medians; whiskers show the minimum and maximum values. A version of these data analyses with complete dataset intervals is given in Supplementary Figure 10.

density, and hygiene, complicates the identification of specific factors linked to antibiotic resistance, as indicated in the two recent molecular-based global studies described above (Hendriksen et al., 2019; Pärnänen et al., 2019). It is possible that water temperature might be more informative in deciphering such trends and we recommend future studies to couple CFU-related data analyses with water temperatures together with air temperatures.

Average defined daily doses (DDD) of β-lactams per 1000 inhabitants in European countries were compared to CFU counts in influents using linear regression analysis, but the tests failed to differentiate correlations between DDD values and coliforms, or between DDD values and CTX-R coliforms, therefore this parameter was considered to be not informative in this study (data not shown). However, it should be noted that DDD values used here reflect general antibiotic usage trends of entire countries and not necessarily the local areas that feed a given WWTP. Previous studies by Collignon et al. (2018) likewise found that annual average β-lactam antibiotics usage in individual communities may not be a reliable predictor of resistance levels of fecal coliforms in wastewater. This may be due to the low resolution of national antibiotic consumption data and may be resolved if coupled with accurate local surveys on antibiotic prescriptions in the area served by a given WWTP, as shown by Caucci et al. (2016). Together with β-lactam antibiotics usage, the contribution of hospital effluents to CTX-R coliforms relative abundance observed in WWTPs' sewage was evaluated comparing (i) the ratio between the number of beds equivalent and population served, to (ii) the observed CTX-R coliforms relative abundance in the influents. From the 17 WWTPs analyzed where data were available (Supplementary Table 1), no correlation was observed (slope not significantly different from 0; Supplementary Fig. 5). The most plausible explanation for these observations is that antibiotic resistance dynamics are dictated by complex ecological interactions that may not be explained solely based on the use of antibiotics in clinical settings or even on the occurrence of antibiotic residues in wastewater (Novo et al., 2013; Varela et al., 2014). Other factors related to the individual wasterwater network at catchement points might further influence such diversity (e.g. volume of flow, combined or separated sewer systems, etc.) (Choi et al., 2018).

3.2. CTX-R coliform removal in WWTPs

The removal of coliforms and CTX-R coliforms was evaluated in WWTPs where both influent and effluent samples were available. Overall, from all sampling dates, a total of 220 count values for colifoms and 215 count values for CTX–R coliforms were compared between influents and effluents. Globally, the average abatement of both coliforms and of CTX-R coliform populations in secondary (CAS and MBR) effluents was 2.3 ± 1.2 log units. MBR treatments showed significantly higher coliform removal capacity when compared to CAS treatments ($p < 0.0001$, mean 5.8 ± 0.6 and 2.1 ± 0.8 log removal for MBR and CAS, respectively). The higher removal observed for MBRs is likely provided by the membranes, especially in the case of ultrafiltration (CYP WWTP), where small pore-size, contributes to the retention of coliforms due to size exclusion and cell-colloid interactions (Schwermer et al., 2017). However, the dataset contained only eight sampling points from MBR whereas CAS accounted for 140 sampling points.

The data from the seven WWTPs that included a final disinfection step were specifically analyzed to evaluate the abundance and removal dynamics of CTX-R coliforms along the treatment continuum (influent, secondary effluent, and disinfected effluent). Overall multiple sampling dates (1st and 2nd campaign combined) from these seven investigated WWTPs contributed to assemble 23 full continuum profiles. All these WWTPs applied CAS treatment combined with one of the following disinfection treatments: chlorination ($n = 2$), UV radiation ($n = 3$), chlorination and UV radiation ($n = 1$) or ozonation ($n = 1$). In these WWTPs, concerning the raw sewage, the CAS treatment reduced the coliforms and CTX–R coliform loads by approximately 2 log-units, whereas average log removal in the final disinfected effluents was 4.4 ± 2 for coliforms and 3.5 ± 1.4 for CTX-R coliforms. However, we observed a high data dispersion of coliforms and CTX-R coliforms loads in the final effluents (Supplementary Figure 6). Indeed, the abundance of CTX-R coliforms in all disinfected effluents analyzed were below the LOQ in 28 sampling instances (35% of total disinfected final effluent samples analyzed).

The scope of secondary treatment removal of coliforms and CTX-R coliforms was investigated as a function of WWTP regional temperature to evaluate a possible correlation between removal efficiency and temperature. Such an association was evaluated in both percentage removal relative to influent levels and absolute log-units removal of CTX-R coliforms choosing two aerials temperature ranks, < 5 °C and > 15 °C (Supplementary Figure 7 and Supplementary Figure 8). Overall, no significant differences were observed between the two groups following secondary biological treatment; however, in geographical locations where temperature was below 5 °C, we identified a greater number of WWTPs with removal efficiencies of < 95% of resistant coliforms (Supplementary Figure 7). Specifically, the lowest efficiencies were observed for POL2, EST10, DEU1, EST2, GBR4, POL3, EST5, AUT2, and EST4, suggesting that these low removal efficiencies are not nationally or geographically associated, and can be eventually sporadic failures of the systems, potentiated or not by lower temperature. Similar observations from previous studies had also showed that higher ambient temperatures and correlate with increased activated sludge removal rates of coliforms (Miranzadeh et al., 2013). It should be noted however, that other WWTPs with higher removal efficiency were present in the same regions. Furthermore, temperature in secondary aeration tanks can be substantially different from air temperature, therefore the climate may not be a strong predictable indicator of removal efficiency.

3.3. CTX-R coliforms in discharged effluents

WWTP effluents are either discharged into natural water bodies such as streams, rivers, lakes, seas, oceans, or used for irrigation of land. In both scenarios, antibiotic–resistant bacteria (and specifically human commensals or pathogens) can potentially be introduced into the urban water cycle and food webs, subsequently leading to increased human exposure. Stakeholders routinely monitor fecal coliforms to assess contamination in effluent-receiving recreational water bodies, and treated wastewater used for irrigation. However, these indicators do not provide insight into the potential contribution of effluents to antibiotic resistance dissemination, which can have substantial environmental

and epidemiological significance (Berendonk et al., 2015; Manaia, 2017). The advantage of the microbiological method described in this study is that it can be adopted with only minor variation by existing national and regional surveillance programs, with minimal need for additional infrastructure or professional requirements, and with minimal added costs.

A total of 244 individual effluent samples were analyzed from both sampling campaigns. Coliforms and CTX-R coliform levels in the analyzed WWTP effluents in the first and second campaigns are shown in Fig. 2B, and the abundance of CTX-R coliforms in individual WWTPs based on the geographic region is shown in Fig. 5. CTX-R coliform abundance in these effluents was highly variable, ranging from values below the LOQ to values exceeding 10^3 CFUs mL^{-1}.

The analysis of the effluent samples with high CTX-R coliforms counts did not reveal noticeable geographic trends, suggesting that these values may be dependent on local WWTP characteristics and performance, although more robust surveillance of a broader range of WWTPs is required to validate this observation. One exception was the Pune WWTP analyzed in this study, which had the highest influent and effluent resistance levels in both absolute and relative abundance in both campaigns. While one WWTP from India is surely not enough to reveal geographic trends, the high influent CTX-R coliforms levels observed therein may be associated with multiple factors including non-regulated antibiotic consumption, contamination from local antibiotic production facilities that may promote the selection of resistance genes. India is reported as one of the largest consumers of antibiotics (Laxminarayan and Chaudhury, 2016) and previous studies had linked fecal coliforms to high levels of ESBL levels in WWTPs in the country (Lamba and Ahammad, 2017). Combined with fragile sanitary infrastructure, the above-mentioned factors might significantly contribute to the observed values (Bengtsson-Palme et al., 2014). Of note, comparatively higher temperatures of India during samplings could have also contributed to the higher coliforms and CTX–R counts observed (in accordance with MacFadden et al., (2018)) possibly due to faster bacterial growth at higher temperatures (Marano and Prakash personal communication). On the other hand, such simplistic inference might not be applied to other countries where a high abundance of CTX-R coliforms was also measured in the treated effluent, such as the WWTPs in Poland, where regulations follow EU requirements. Despite various attempts of geographical sorting indeed, apart from Poland, we could not identify any clear links between CTX-R coliform effluent levels and specific countries. This was evident for example for Spain (n_{WWTP} = 7), UK (n_{WWTP} = 5), Italy (n_{WWTP} = 4), and in particular Estonia (n_{WWTP} = 12) where different WWTPs exhibited both high and low CTX–R coliforms effluent values.

3.4. Implications and potential standards for discharged CTX-R coliforms in receiving environments

This study provides data that can facilitate the development of urgently required criteria for the permitted levels of CTX-R coliforms in effluents and various water reuse scenarios. Existing fecal coliform standards for wastewater recognize the potential threat that wastewater effluent poses to human health, although criteria for 'safe' levels of fecal indicators vary greatly between regions and use. For example, in California and Israel, the fecal coliform limits are 2.2 and 10 CFU per 100 mL, respectively (EPA, 2012). In Greece and Cyprus, these are 100 CFU per 100 mL for fecal coliforms, whereas the World Health Organization (WHO) standards for water re-use for irrigation are 1,000 CFU per 100 mL for fecal coliforms (Blumenthal et al., 2000). Similar criteria exist for discharge into water bodies intended for recreational use. For example, the US Environmental Protection Agency recommends a limit of 200 CFU per 100 mL of fecal coliforms in rivers and streams used for swimming (U.S. EPA, 1976). While most of the effluent samples contained low levels of CTX–R coliforms, it is troubling that 37% (89 out of 243 individual sampling points) exceeded the WHO

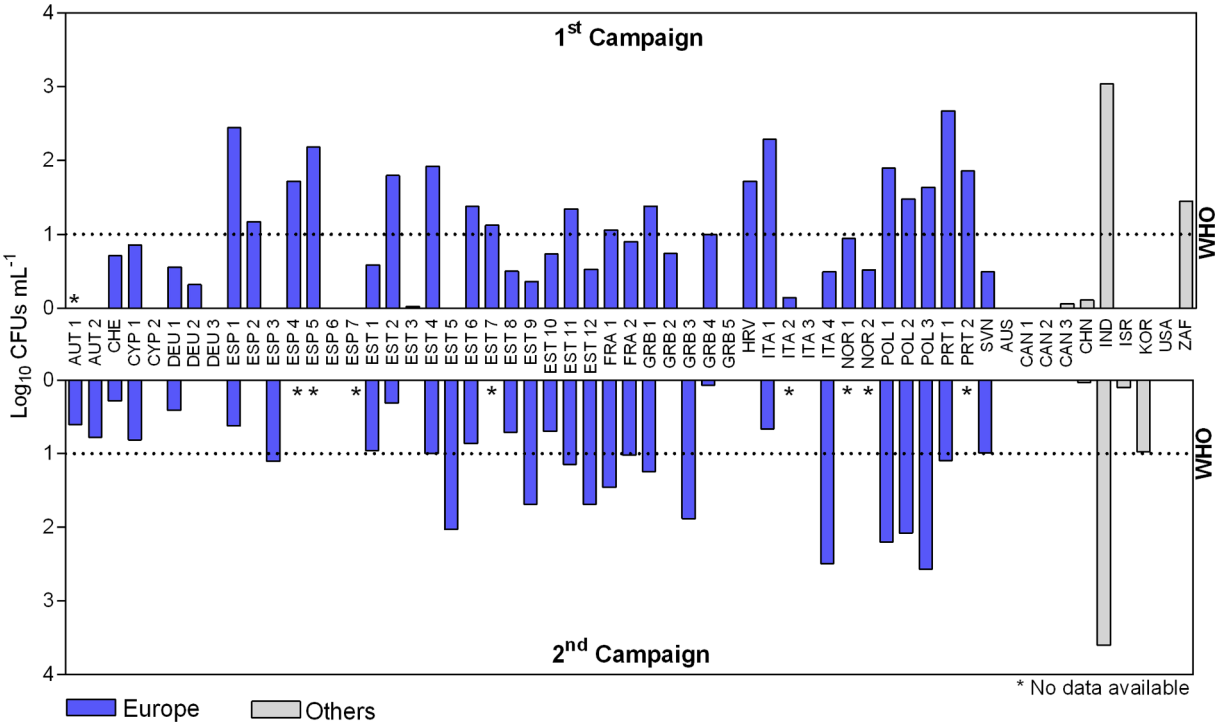

Fig. 5. Average CTX-R coliforms mL^{-1} in WWTPs' effluents (country-code + WWTP's assigned number from the same country). Fifty-seven WWTPs sampled in the first campaign (top) and forty-eight in the second campaign (bottom). The dotted line shows the WHO limits for water reuse regulation referred to Escherichia coli (CFUs mL^{-1}) log converted. Australia (AUS); Austria (AUT); Canada (CAN); China (CHN); Croatia (HRV); Cyprus (CYP); Estonia (EST); France (FRA); Germany (DEU); India (IND; Pune); Israel (ISR); Italy (ITA); Republic of Korea (KOR); Norway (NOR); Poland (POL); Portugal (PRT); Slovenia (SVN); South Africa (ZAF); Spain (ESP); Switzerland (CHE); United Kingdom (GRB); United States (USA). Asterisks indicated missing participation in the sampling campaign.

cutoff formulated for fecal coliforms (resistant and not; Fig. 5). Of note, as discussed above, whilst the hereby proposed method detects also coliforms other than *E. coli* (conventionally the most used fecal coliform indicator), *E. coli* still represent up to 50% of the pool of coliforms detected on mFC Agar plates incubated at 37 °C. If one considers that an average large WWTP (e.g. 50,000 person equivalent) can release more than 10^7 L per day of effluent, we estimate that under suboptimal treatment conditions (CTX–R coliforms $>$ 10 CFU mL^{-1} referring to the above mentioned WHO indicated threshold for fecal coliforms), up to 10^{11} CTX-R coliforms may be released daily into effluent receiving rivers. Additionally, if we consider that under Mediterranean climate conditions seasonal irrigation can exceed 1 m^3 of water per square meter of soil, this would mean that under the same assumptions each square meter of soil could potentially receive 10^8 CTX-R coliforms per season. While many countries have developed methods for optimizing the irrigation of edible crops, long-term exposure to antibiotic resistance determinants still might increase the risks of systematic transmission of these determinants throughout natural environments. Different types of downstream environments might present different permissiveness in terms of antibiotic-resistant bacteria dissemination and persistence, and release of antibiotic-resistant bacteria into surface water bodies can have a different and broader impact than the release in soil (Eckert et al., 2018; Leonard et al., 2018; Munck et al., 2015). Future studies should seek to ascertain whether antibiotic-resistant bacteria in these different environments are transmitted to humans, as well as to describe the impacts on human health resulting from such exposures. Additionally, they should characterize the genetic and phenotypic diversity of CTX-R coliforms and evaluate their capacity to persist in downstream environments (Karkman et al., 2018). Finally, effluents intended for re-use or discharged to bathing waters should be further investigated to assess the presence of pathogens, such as resistant *K. pneumoniae*, and enterohemorrhagic *E. coli* (EHEC) strains (Nguyen and Sperandio, 2012).

4. Conclusion

From the comprehensive analysis conducted here, differential patterns in the distribution of CTX-R coliforms are clear, although the factors influencing the observed differences are yet to be fully elucidated. This approach could be even more informative if such monitoring approaches are adopted to conduct large-scale national, regional and international surveillance projects that target many WWTPs across a given country or region, and if individual WWTPs are robustly monitored as part of routine monitoring campaigns. The global observed discrepancy between the variance of CTX-R coliforms and the total coliforms in the influents worldwide provides evidence that drivers of such variety might subsist. In order to better understand the factors that dictate this scope in WWTPs, future studies should perform more exhaustive analysis of specific WWTPs and link them to a range of *meta*-parameters related to the WWTP's surrounding area and the served population over time, in order to elucidate the factors influencing the distribution of CTX-R coliforms, such as β-lactam usage only in the population served by the targeted WWTPs (including hospital and livestock untreated/pre-treated wastewater effluents when relevant) to contextualize the outcomes to the 'big picture' of antibiotic resistance. Collectively, these approaches would facilitate: (i) detection of external factors and selective pressures that potentially contribute to antibiotic resistance levels in WWTPs, (ii) identification of local and global antibiotic resistance trends in WWTPs, (iii) understanding of the effects of quantified emission values of CTX-R coliforms in effluent-receiving environments, and (iv) help identify possible unexpected high peaks increasing the risk of clinical outbreaks when effluent wastewater is discharged into surface water or reused in agriculture.

Cefotaxime-amended chromogenic selective media are increasingly applied in surveillance studies (Snow et al., 2011) and can potentially serve as an alternative to the cefotaxime-amended mFC medium used here; however we recomend the used cefotaxime concentration to be 4 μg mL^{-1}. Given the growing epidemiological relevance of carbapenemase-producing *Enterobacteriaceae*, future surveys targeting WWTPs should concomitantly apply mFC medium amended with imipenem or meropenem, or use commercial available chromogenic media (García-Fernández et al., 2017). Finally, a subset of the isolates obtained should be subject to screening for cefotaxime-resistance genes (*i.e.* those encoding CTX-M enzymes), using PCR and/or whole genome sequencing and comparative analyses of ARGs and MGEs to detect possible geographic patterns.

Credit authorship contribution statement

Roberto B.M. Marano: Investigation, Data curation, Project administration. **Telma Fernandes:** Investigation, Data curation, Formal analysis, Visualization. **Célia M. Manaia:** Conceptualization, Methodology, Supervision, Validation, Resources. **Olga Nunes:** Conceptualization, Methodology, Supervision, Validation, Resources. **Donald Morrison:** Supervision, Validation, Resources. **Thomas U. Berendonk:** Supervision, Resources, Validation. **Norbert Kreuzinger:** Supervision, Resources. **Tanel Tenson:** Resources. **Gianluca Corno:** Supervision, Resources. **Despo Fatta-Kassinos:** Resources. **Christophe Merlin:** Supervision, Resources. **Edward Topp:** Supervision, Resources. **Edouard Jurkevitch:** Supervision, Validation. **Leonie Henn:** Resources, Validation. **Andrew Scott:** Resources. **Stefanie Heß:** Resources. **Katarzyna Slipko:** Resources. **Mailis Laht:** Resources. **Veljo Kisand:** Resources. **Andrea Di Cesare:** Supervision, Resources. **Popi Karaolia:** Resources. **Stella G. Michael:** Resources. **Alice L. Petre:** Resources. **Roberto Rosal:** Resources. **Amy Pruden:** Supervision, Validation, Resources. **Virginia Riquelme:** Resources. **Ana Agüera:** Resources. **Belen Esteban:** Resources. **Aneta Luczkiewicz:** Validation. **Agnieszka Kalinowska:** Validation. **Anne Leonard:** Resources. **William H. Gaze:** Resources. **Anthony A Adegoke:** Resources. **Thor A Stenstrom:** Resources. **Alfieri Pollice:** Resources. **Carlo Salerno:** Resources. **Carsten U. Schwermer:** Resources. **Pawel Krzeminski:** Resources. **Hélène Guilloteau:** Resources. **Erica Donner:** Supervision, Validation, Resources. **Barbara Drigo:** Supervision, Validation, Resources. **Giovanni Libralato:** Resources. **Marco Guida:** Resources. **Helmut Bürgmann:** Resources. **Karin Beck:** Resources. **Hemda Garelick:** Validation. **Marta Tacão:** Resources. **Isabel Henriques:** Resources. **Isabel Martínez-Alcalá:** Resources. **Jose M. Guillén-Navarro:** Resources. **Magdalena Popowska:** Resources. **Marta Piotrowska:** Resources. **Marcos Quintela-Baluja:** Resources. **Joshua T. Bunce:** Resources. **Maria I. Polo-López:** Resources. **Samira Nahim–Granados:** Resources. **Marie-Noëlle Pons:** Resources. **Milena Milakovic:** Resources, Validation. **Nikolina Udikovic-Kolic:** Resources, Validation. **Jérôme Ory:** Resources. **Traore Ousmane:** Resources. **Pilar Caballero:** Resources. **Antoni Oliver:** Resources. **Sara Rodriguez-Mozaz:** Resources. **Jose L. Balcazar:** Resources. **Thomas Jäger:** Validation. **Thomas Schwartz:** Resources. **Ying Yang:** Resources. **Shichun Zou:** Resources. **Yunho Lee:** Resources. **Younggun Yoon:** Resources. **Bastian Herzog:** Resources. **Heidrun Mayrhofer:** Resources. **Om Prakash:** Resources, Validation. **Yogesh Nimonkar:** Resources. **Ester Heath:** Resources. **Anna Baraniak:** Validation. **Joana Abreu-Silva:** Validation. **Manika Choudhury:** Validation. **Leonardo P. Munoz:** Validation. **Stela Krizanovic:** Resources, Validation. **Gianluca Brunetti:** Validation. **Ayella Maile-Moskowitz:** Validation. **Connor Brown:** Validation. **Eddie Cytryn:** Conceptualization, Methodology, Supervision, Validation, Resources.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106035.

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A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants

		1st campaign (December 2016 – February 2017)				2nd campaign (May 2017 – October 2017)			
Indicator Bacteria (CFU mL^{-1})	SOURCE	Mean	SD	Min	Max	Mean	SD	Min	Max
Coliforms	Inflow	1.3E+06	4.9E+06	3.3E+03	3.7E+07	1.8E+06	6.2E+06	3.3E+04	4.8E+07
	Outflow	1.4E+04	5.6E+04	0.0E+00	5.6E+05	1.1E+04	3.6E+04	0.0E+00	2.1E+05
CTX-R Coliforms	Inflow	1.5E+04	5.4E+04	0.0E+00	5.8E+05	1.2E+05	7.6E+05	3.7E+00	6.7E+06
	Outflow	3.2E+02	2.3E+03	0.0E+00	3.0E+04	2.2E+02	8.5E+02	0.0E+00	5.0E+03

Supplementary Table 1. Abundance of total and cefotaxime-resistant (CTX-R) coliforms in influents and effluents of all sampled WWTPs.

Country	Identification method	Primer name	Sequence	Reference
PRT 1	16S rRNA	27-F	GAGTTTGATCCTGGCTCAG	(Miller et al., 2013)
		1492-R	TACCTTGTTACGACTT	(Frank et al., 2008)
POL 2	16S rRNA	27-F	GAGTTTGATCCTGGCTCAG	(Miller et al., 2013)
		1492-R	TACCTTGTTACGACTT	(Frank et al., 2008)
DEU 1	16S rRNA	27-F	GAGTTTGATCCTGGCTCAG	(Miller et al., 2013)
		517-R	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
USA	16S rRNA	515-F	GTGCCAGCMGCCGCGGTAA	(Tanner et al., 2000)
		926-R	CCGYCAATTYMTTTRAGTTT	(Muyzer et al., 1995)
GRB 4	MALDI-TOF	-	-	-
HRV	MALDI-TOF	-	-	-
ISR	MALDI-TOF	-	-	-
AUS	MALDI-TOF	-	-	-

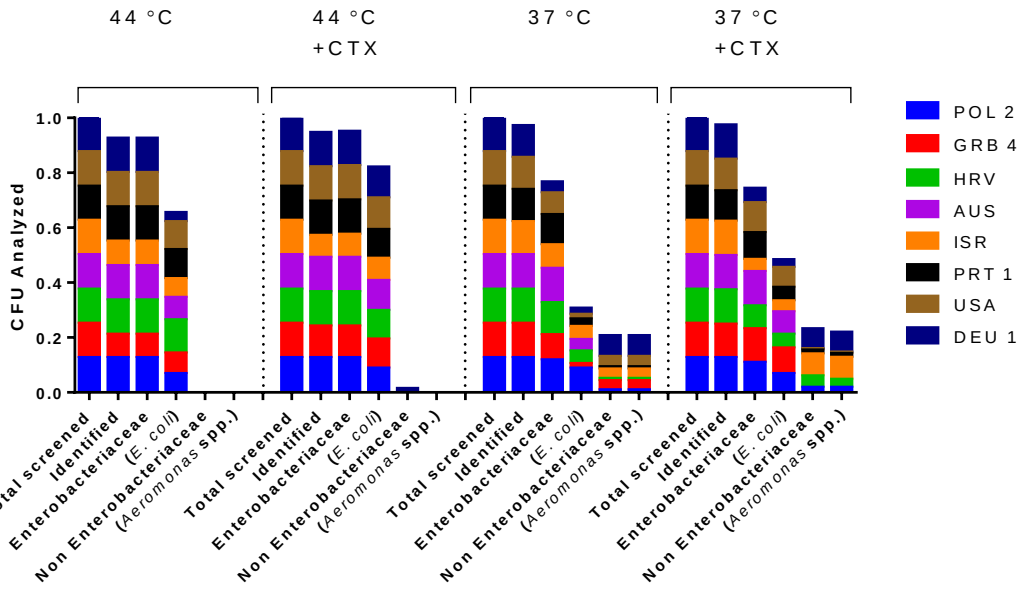
Supplementary Table 2. **Bacterial validation of detected coliforms** (blue CFU isolates on mFC Agar) performed by the eight participating laboratories, Identification was conducted either by Sanger sequencing of 16S rRNA gene amplicons or by MALDI-TOF.

Library assembly

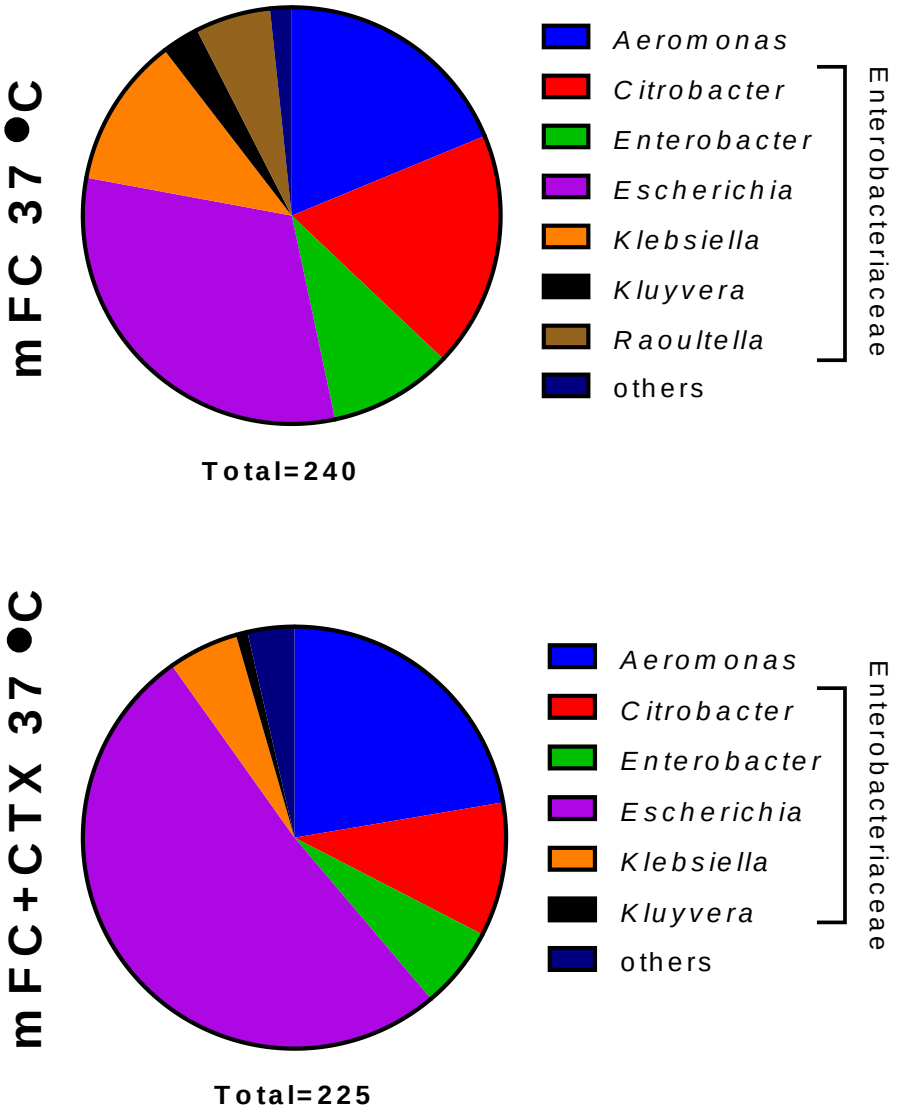
To validate the efficacy of the proposed protocol an additional sampling campaign was conducted by eight of the consortium participants. Grab samples (1 L) taken from secondary effluents (only), were filtered and plated (in six replicates) as described in section 2.4. Filter membranes plated on mFC agar supplemented with/without cefotaxime (CTX) were then split into two parallel batches and incubated at 37 °C or 44 °C for 24 hours (triplicates for each treatment at both temperatures). Subsequently, from plates within the countable CFU range (10-80) from each of the four conditions, 30 randomly selected blue colonies were streaked for isolation on new plates and incubated at the same conditions 24 h. Finally, following the isolation procedure, four libraries of 30 isolates each from the four described conditions were assembled on partitioned LB agar plates, incubated for 24 h at 37 °C and screened within 1 day for taxonomic characterization by colony-PCR using universal 16S rRNA primers and sequencing, or MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) mass spectrometry characterization, as described below. Data from 6 of the 8 participating groups were used to compare the abundance of CTX-resistant *E. coli* when incubating at 37 °C relative to those quantified at 44 °C.

Library screening and identification methods

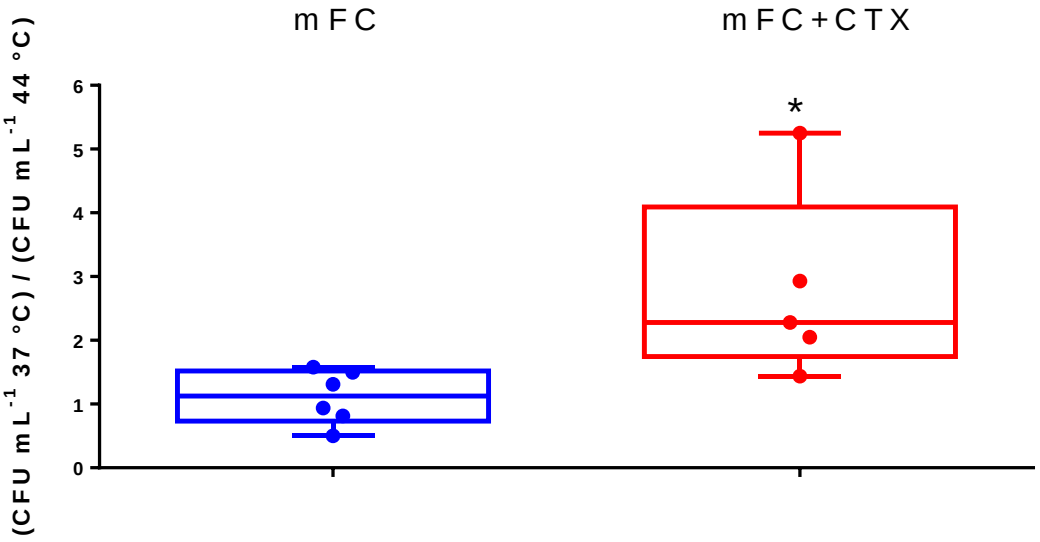
Identification of blue colonies from the mFC agar library assembly described above was achieved by either 16S rRNA amplicon sequencing or MALDI-TOF, according to the available facilities each group had access to. PCR of 16s rRNA genes and subsequent sequencing was carried out using internally validated protocols with universal bacterial primers of choice (reported in Supplementary Table 3); generated high-quality sequences were screened using the NCBI blastn pipeline with default parameters. MALDI-TOF users referred to internally established protocols, and internally available databases for identification. Taxonomic results were then summarized at genus or family level.



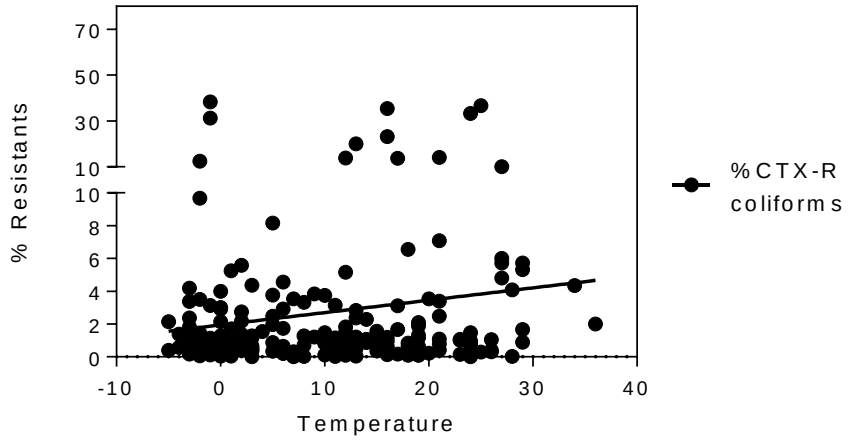
Supplementary Figure 1. Cumulative distribution of taxonomically characterized CFUs from screened libraries. Each participant group (country abbreviation indicating in figure legend) screened four types of libraries (~30 each) each randomly assembled from blue CFU isolates retrieved from filters plated on mFC agar +/- cefotaxime (CTX) respectively incubated for 24 h at 37 °C and 44 °C.



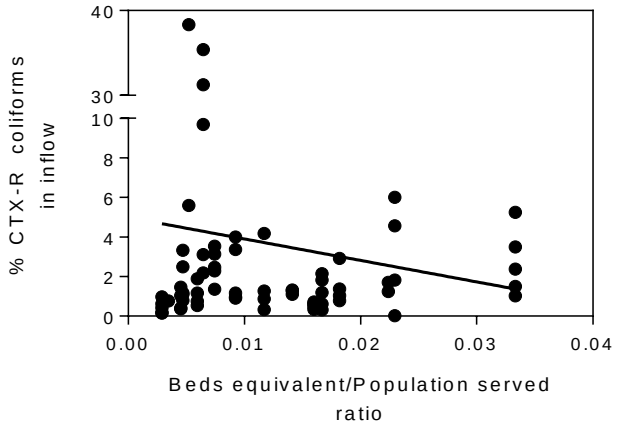
Supplementary Figure 2. Distribution of genera with >3% abundance over a pool of identified isolates retrieved from screened libraries of mFC Agar +/- cefotaxime(CTX) incubated for 24 h at 37 °C from 8 participant groups.



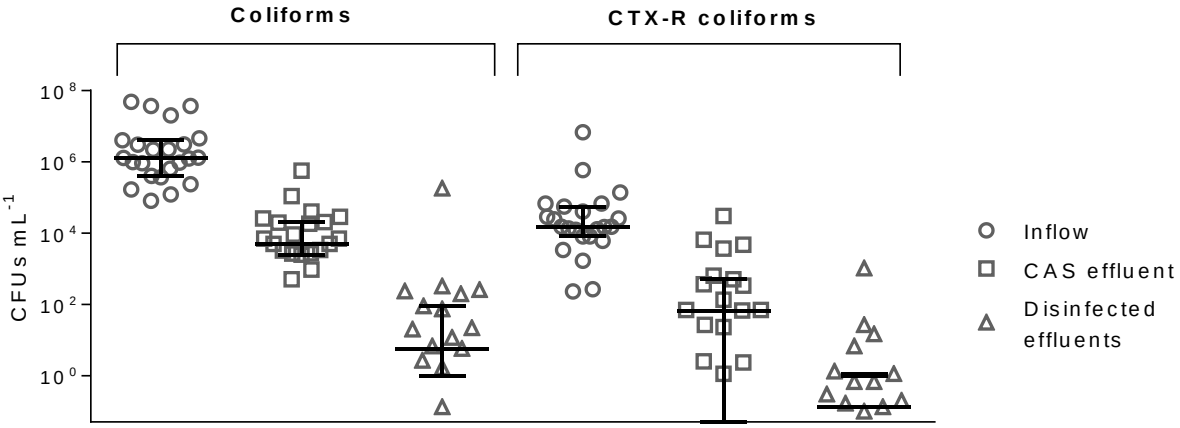
Supplementary Figure 3. Comparison of *Escherichia coli* abundance on mFC Agar incubated at 37 °C *vs.* 44 °C from six WWTPs from the validation experiment (Supplementary Figure 6). **Left box-plot**: Total 37 °C *vs.* 44 °C *E. coli* estimated abundance ratio. **Right box-plot**: Cefotaxime-resistant (CTX-R) incubated at 37 °C *vs.* 44 °C *E. coli* abundance ratio. On average, the ratios of CTX-R *E. coli* recovered on mFC Agar+CTX was higher ($p<0.05$) when incubating at 37 °C than at 44 °C than the same ratios from the same samples incubated on mFC Agar at 37 °C or 44 °C



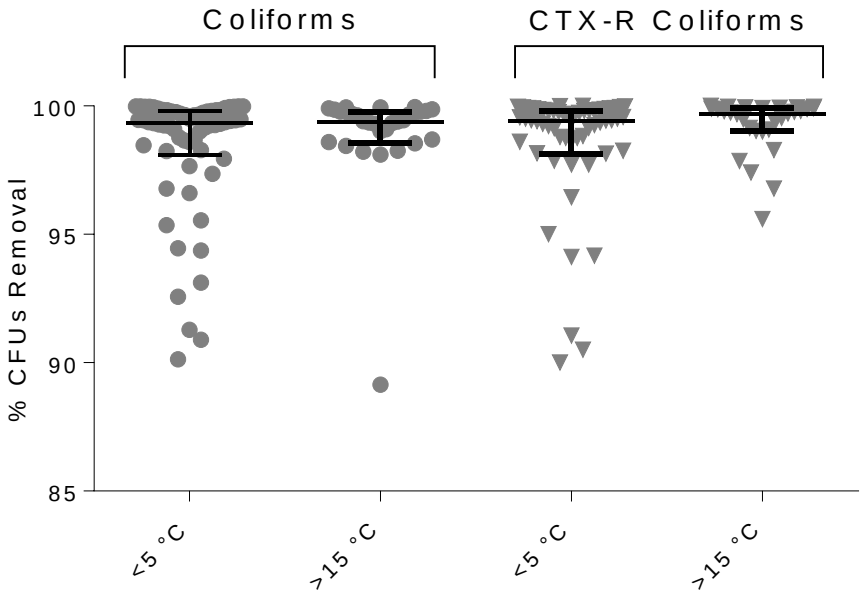
Supplementary Figure 4. Relative abundance of cefotaxime-resistant (CTX-R) coliforms in influents from all sampling dates of all WWTPs as a function of the mean temperature from the two seasonal sampling campaigns. 0-10 % and 10-80 % intervals are shown. Plotted lines refer to the fitted linear model; results suggest little evidence of an association between temperature and % CTX-R coliforms ($R^2<0.01$, $p=0.18$).



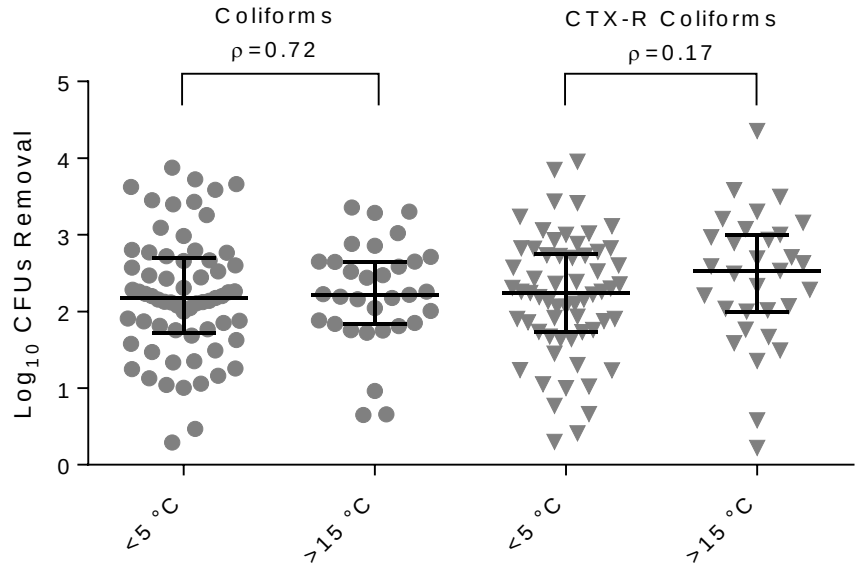
Supplementary Figure 5. **Abundance of cefotaxime-resistant (CTX-R) coliforms in WWTPs inflow as a function of bed equivalents and population served ratios.** Bed equivalents of hospitals discharging effluents to 17 WWTPs from the two sampling campaigns are normalized to the related equivalent population served and compared to the related abundance of CTX-R coliforms in the inflows. 0-10 % interval and 30-40 % intervals are shown. Plotted lines show the fitted linear model. $R^2<0.1$; slope not significantly different from 0.



Supplementary Figure 6. Distribution of coliforms and cefotaxime-resistant (CTX-R) coliforms in influents, secondary (conventional activated sludge , CAS), and disinfected treated wastewater from seven WWTPs (total sampling dates from 1st and 2nd sampling campaigns=23); median with interquartile range highlighted.



Supplementary Figure 7. Removal of coliforms and cefotaxime-resistant (CTX-R) coliforms by conventional activated sludge (CAS) treatment as a function of temperature. Values from all samples were divided into two ranks (<5 °C and >15 °C); median and with interquartile range highlighted. Coliforsm < 5°C, n=65; coliforms >15 °C, n=32; CTX-R coliforms < 5°C, n=61; CTX-R coliforms > 15 °C, n=31.



Supplementary Figure 8. Removal of coliforms and cefotaxime- resistant (CTX-R) by CAS treatment as a function of temperature. Values from all samples were divided into two ranks (<5 °C and >15 °C); median and with interquartile range highlighted. p values refer to a Mann-Whitney U-test. Coliforms < 5°C n=65; coliforms > 15 °C n=32; CTX-R colirms < 5°C n=61; CTX-R coliforms > 15 °C n=31.

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