UNIONE EUROPEA
Fondo Sociale Europeo

Ministero dell'Università e della Ricerca

PON RICERCA E INNOVAZIONE

REACT EU

UNIVERSITÀ DEGLI STUDI DI TRIESTE

Università Ca'Foscari Venezia

UNIVERSITÀ DEGLI STUDI DI TRIESTE
UNIVERSITÀ CA' FOSCARI VENEZIA

XXXVII CICLO DEL DOTTORATO DI RICERCA IN CHIMICA

Risultati conseguiti con il finanziamento ottenuto a valere sull'Asse IV del PON Ricerca e Innovazione 2014-2020 "Istruzione e ricerca per il recupero – REACT-EU"

Development and application of procedures for environmental bioaerosol characterization and assessment of microbial abatement technologies

Settore scientifico-disciplinare: **CHEM-01/B**

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Prof. Pierluigi Barbieri

ANNO ACCADEMICO 2023/2024

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Abstract

Bioaerosols are dynamic atmospheric components of biological origin – including bacteria, viruses, pollen, and fungi – shaped by both natural processes and anthropogenic factors. They exert considerable influence on agriculture, climate, ecosystems, and public health, as underscored by the airborne transmission of pathogens such as SARS-CoV-2. Despite its importance, research on bioaerosol remains at an early stage, largely due to the lack of standardized methodologies for sampling and analysis. This methodological gap not only hinders comprehensive understanding but also compromises the comparability of findings between different studies. Current bioaerosol research is constrained by inconsistent approaches and the inherently low biomass of microbial particles in the atmosphere. Sampling techniques are typically classified as passive (gravitational settling) or active (mechanical air movement), each with specific advantages or drawbacks. Passive methods are simple and cost-effective but lack quantitative precision, whereas active methods allow detailed analysis but may compromise microbial viability. Furthermore, environmental variability and the dynamic behavior of bioaerosols further complicate their study. Culture-dependent and culture-independent techniques, such as DNA metabarcoding and metagenomics, offer new opportunities to explore bioaerosol diversity. However, these approaches are also affected by biases, such as the challenges of low DNA yield from bioaerosol samples.

This thesis aims to overcome these challenges by refining sampling methodologies, developing innovative laboratory protocols, and integrating omics approaches to support more reliable and comparable bioaerosol studies. By posing attention on both controlled laboratory conditions and field environments this thesis seeks to advance the knowledge of bioaerosol dynamics, improve data comparability, and inform public health and environmental management strategies.

The five studies illustrated in this thesis, collectively address fundamental aspects of bioaerosol research, including air disinfection and characterization of various critical environments (e.g., hospitals and wastewater treatment plants), emphasizing the challenges and recent developments.

Starting the doctoral project in the COVID-19 pandemic period, in the context of SARS-CoV-2, a novel protocol was proposed to evaluate the Viral Filtration Efficiency (VFE) of NanoAg-coated endonasal filters. The experimental setup involves a SARS-CoV-2 aerosol transmission chamber, a BLAM aerosol generator, and a Biosampler. Residual infectivity is proposed to be assessed from aliquots of liquid collecting bioaerosol, sampled with and without endonasal filters. Cytopathic effect as plaque formation and viral proliferation, assessed via qRT-PCR (quantitative reverse transcription polymerase chain reaction) on Vero E6 cells, can be monitored for up to 7 days post-inoculation. The

apparatus proved suitable for advanced aerosol contaminated studies and infection prevention applications.

The second study presented in this thesis introduces an innovative air disinfection device that integrates UV-C LEDs with a highly reflective optical material called Porex, along with an aerodynamic filter developed by Computer-Aided Design. Bioaerosol removal efficiency was tested in a laboratory setup using an impinger nebulizer, a cylindrical aerosol chamber, and the SKC BioSampler. *Escherichia coli* (BL21-DE3) served as a model organism, and optimization of operational settings was achieved via sequential D-optimal designs. The device exhibited high bioaerosol inactivation efficiency under optimized conditions. This air disinfection device is therefore a promising and cost-effective solution for enhancing indoor air quality.

With the aim of studying and optimizing effective bioaerosol characterization and risk reduction procedures in real-world scenarios, a separate study evaluated bioaerosol dynamics during dental procedures at the Maxillofacial Surgery and Dentistry Clinic of the Maggiore Hospital in Trieste (Italy). Sampling was conducted with Tryptic Soy Agar (TSA) Petri dishes placed in the dentistry units to capture bioaerosol particles. Colony counts were analyzed post-incubation, and microbial identification was performed using the 16S rRNA metabarcoding technique. The findings revealed proximity-dependent bioaerosol gradients, with significantly higher deposition rates during operating hours. Identified colonies were primarily *Staphylococcus* species, including opportunistic pathogens. The study emphasized the importance of enhanced ventilation and strategic clinic design to mitigate bioaerosol-related risks.

Lack of standardization in bioaerosol field sampling addressed a further study, where bioaerosol biodiversity was examined in the wastewater treatment plant in Trieste (Italy) employing three sampling systems: gelatin filtration, swirling aerosol collection, and condensation growth tubes. Untreated wastewater samples were also analyzed, and bacterial diversity from all water and bioaerosol samples was assessed through the 16S rRNA gene metabarcoding technique. Results indicated significant variability in bacterial community composition based on the sampling method used, with the condensation growth tubes sampling system capturing the richest bacterial genera diversity. The study highlights that methodological standardization is crucial for accurate qualitative and quantitative comparisons.

Lastly, the same wastewater treatment plant served as site for a second study aimed at assessing the presence of antimicrobial molecules and antibiotic resistance genes (ARGs), as well as microbial community shifts, in both influent and effluent waters through metagenomics and chemical analyses (as it is addressed by recent new wastewater treatment plant EU Directive). Results showed effective removal of several antimicrobial molecules, whereas persistent compounds like fluconazole and

ciprofloxacin exhibited minimal reduction. Although metagenomics revealed significant ARGs reductions, residual resistance genes remained in effluents. The study underscores that while the plant effectively mitigates contamination, advanced treatment methods are required to definitively eliminate persistent antimicrobial molecules and ARGs.

Riassunto

I bioaerosol sono componenti atmosferici dinamici di origine biologica, come batteri, virus, pollini e funghi. Essi sono influenzati da processi naturali ma anche da fattori antropogenici. Il loro impatto sulla biosfera è rilevante, in quanto possono influenzare l' agricoltura, il clima, gli ecosistemi e la salute pubblica, con la trasmissione aerea di patogeni come il SARS-CoV-2. Nonostante la sua rilevanza, la ricerca sul bioaerosol si può intendere ancora agli esordi, principalmente a causa dell' assenza di metodologie standardizzate per il campionamento e l' analisi. Questo non solo limita una conoscenza approfondita e complessiva sull' argomento ma rende anche difficilmente confrontabili i risultati ottenuti da studi diversi. Le tecniche di campionamento, classificate in passive (deposizione delle particelle per gravità) e attive (movimento meccanico attivo dell'aria attraverso un campionatore), presentano ciascuna vantaggi e limiti. I metodi di campionamento passivo sono semplici e poco costosi ma mancano di caratterizzazione quantitativa; i metodi di campionamento attivo, invece, consentono un campionamento più controllato ma spesso compromettono la vitalità microbica. Inoltre, la variabilità dei fattori ambientali e la natura dinamica del bioaerosol complicano ulteriormente la ricerca in questo campo.

Le tecniche che non si avvalgono di metodi di coltura per lo studio del bioaerosol, quali il DNA *metabarcoding* e la *metagenomica*, offrono nuove prospettive per lo studio della diversità dei microorganismi aerodispersi. Tuttavia, questi approcci non sono esenti da *bias*, come ad esempio le difficoltà legate alla bassa resa di DNA nei campioni di bioaerosol.

La presente tesi ha come scopo quello di affrontare tali tematiche e criticità, migliorando la conoscenza sulle attuali metodologie di campionamento del bioaerosol. Questo grazie allo sviluppo di protocolli di campionamento e di analisi di laboratorio innovativi, nonché all'integrazione di approcci "omici" per una migliore comparazione dei risultati. In particolare, sono stati condotti studi sia in ambienti controllati di laboratorio che in campo, con l' obiettivo di approfondire la comprensione delle dinamiche del bioaerosol, migliorare la comparabilità dei dati e fornire indicazioni utili per la gestione della salute pubblica e ambientale.

I cinque studi illustrati in questa tesi affrontano collettivamente aspetti fondamentali della ricerca sul bioaerosol, come la disinfezione dell' aria e la caratterizzazione di diversi ambienti critici, tra cui ospedali e impianti di trattamento delle acque reflue, ponendo l'accento sulle sfide e sui progressi in questi ambiti.

Nel contesto del SARS-CoV-2, è stata proposta una procedura innovativa in ambiente controllato di laboratorio per la valutazione dell' efficienza di filtrazione virale (VFE) di filtri endonasali rivestiti con NanoAg. Il *setup* sperimentale comprende una camera di trasmissione di aerosol contenente

SARS-CoV-2, un aerosolizzatore BLAM e un BioSampler. L' infettività residua è analizzata prelevando aliquote di liquido contenente il bioaerosol, campionato in presenza e in assenza di filtri endonasali. Si valuta quindi l' effetto citopatico come formazione di placche e la proliferazione virale tramite qRT-PCR (reazione a catena della polimerasi con trascrizione inversa quantitativa) su cellule Vero E6, fino a 7 giorni dal momento dell'inoculo. Il *setup* si è dimostrato idoneo a studi avanzati su aerosol contaminati da microorganismi patogeni e ad applicazioni nella prevenzione delle infezioni.

Il secondo studio presentato in questa tesi introduce un dispositivo innovativo per la disinfezione dell'aria, che combina LED UV-C e un materiale ottico altamente riflettente denominato Porex, in associazione ad una specifica struttura aerodinamica. L' efficienza di rimozione del bioaerosol è stata valutata in ambiente controllato di laboratorio, utilizzando un nebulizzatore *impinger*, una camera cilindrica di diffusione dell' aerosol e un campionatore SKC BioSampler. Come organismo modello è stato impiegato *Escherichia coli* (BL21-DE3), e la messa a punto delle variabili sperimentali è stata realizzata tramite *D-optimal design* sequenziali. I risultati hanno evidenziato un' elevata efficienza di inattivazione del bioaerosol in condizioni ottimali. Tale dispositivo di disinfezione dell' aria si propone pertanto come soluzione efficiente ed economica per migliorare la qualità dell' aria negli ambienti *indoor*.

Il terzo studio coinvolge invece l' ambito clinico, ed ha come scopo quello di indagare le dinamiche di generazione del bioaerosol durante le procedure odontoiatriche nella Clinica di Chirurgia Maxillo-Facciale e Odontoiatria presso l'Ospedale Maggiore di Trieste (Italia). Il campionamento è stato realizzato utilizzando piastre Petri contenenti *Tryptic Soy Agar* (TSA) collocate nei riuniti della clinica, allo scopo di catturare le particelle di bioaerosol disperse nell'ambiente. Le colonie generate su piastra sono state contante e analizzate seguendo la tecnica del DNA *metabarcoding* del gene 16S rRNA. I risultati hanno rivelato gradienti di bioaerosol dipendenti dalla prossimità del paziente, con tassi di deposizione significativamente più elevati durante le ore operative. L'identificazione batterica ha rivelato una dominanza di specie appartenenti al genere *Staphylococcus*, inclusi patogeni opportunisti. Lo studio sottolinea l'importanza di una ventilazione adeguata negli ambienti *indoor* e prevedere soluzioni progettuali adeguate a mitigare i rischi associati al bioaerosol in ambienti odontoiatrici.

In un ulteriore studio effettuato in campo, è stata esaminata la biodiversità del bioaerosol presente presso l' impianto di trattamento delle acque reflue a Trieste (Italia), attraverso l' impiego di tre sistemi di campionamento diversi: filtrazione, raccolta di bioaerosol con modalità *swirling* e tubi di condensazione. Insieme ai campioni di bioaerosol, sono stati analizzati anche campioni di acque reflue, e la diversità batterica è stata determinata mediante la tecnica del DNA *metabarcoding* del gene 16S rRNA. I risultati hanno evidenziato una marcata variabilità nella composizione delle

comunità batteriche a seconda del metodo di campionamento utilizzato; in particolare, il sistema a tubi di condensazione ha catturato la maggiore biodiversità a livello di generi batterici. Questo studio ha evidenziato come la standardizzazione metodologica sia essenziale per analisi quantitative e qualitative affidabili.

Infine, lo stesso impianto di trattamento delle acque reflue è stato oggetto di un ulteriore studio, finalizzato a valutare la presenza di molecole antimicrobiche e geni di resistenza agli antibiotici (ARG), nonché le variazioni delle comunità microbiche, sia nelle acque reflue che in quelle effluenti dell'impianto. Lo studio è stato svolto utilizzando analisi metagenomiche e chimiche. I risultati hanno mostrato un' efficace rimozione di diversi antibiotici, mentre alcuni composti più persistenti come il fluconazolo e la ciprofloxacina hanno mostrato riduzioni minime. Le analisi metagenomiche hanno rivelato significative riduzioni degli ARG, pur mettendo in luce la presenza di geni di resistenza residui nelle acque effluenti dell' impianto. Lo studio evidenzia che, sebbene l' impianto sia in grado di ridurre efficacemente la contaminazione chimica e biologica delle acque reflue, sono tuttavia necessari metodi di trattamento avanzati per eliminare in modo definitivo alcune molecole antimicrobiche e ARG, entrambi coinvolti nel fenomeno dell' antibiotico resistenza.

1 Introduction

1.1 Bioaerosol

Bioaerosol is the aerosol containing components of biological origin. Typically, it consists of viruses, bacteria, pollens, fungi, spores, and fragments of plant or animal matter. Therefore, its diameter ranges from 1 nm to 100 µm [1–5] (Figure 1). Bioaerosols are found in the environment as aggregates to water droplets, particulate matter (PM) or as an individual organism [2].

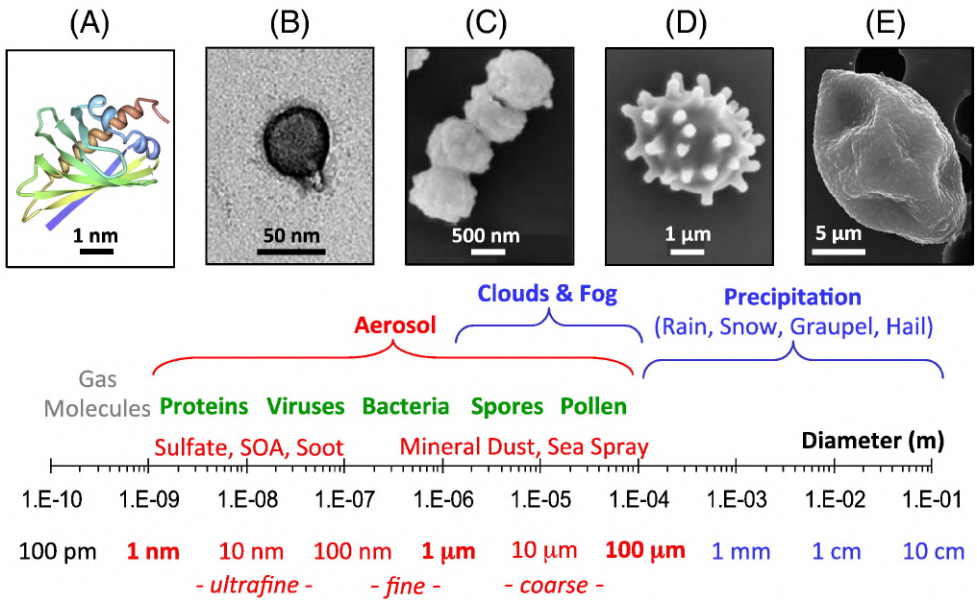

Figure 1. Characteristic size ranges of atmospheric particles and bioaerosols with exemplary illustrations: (A) protein, (B) virus, (C) bacteria, (D) fungal spore, and (E) pollen grain [4,6].

Bioaerosols may originate from natural processes, such as soil erosion, which releases microorganisms attached to soil particles, and plant life cycles, which disperse pollen and spores into the air. Anthropogenic activities also contribute significantly to bioaerosol production: agricultural practices release microbial communities from soil and plant pathogens, wastewater treatment aerosolizes microorganisms, and industrial processes emit biologically laden particulates [7]. Once released from the source, microorganisms can remain airborne for extended periods, with some capable of traveling hundreds to thousands of kilometers depending on atmospheric conditions. For example, studies have documented microbial transport across continents, demonstrating their persistence and potential for long-range dispersion [8]. Studies are also developed on the interaction between bioaerosols and environmental particulate matter [9,10], and this subject is part of a PRIN project in which the undersigned has recently been involved.

The interest in the biogenic fraction of aerosols has increased over the years as various studies highlighted how it can impact different spheres of human life but also more generally of our planet (Figure 2). In fact bioaerosol can impact: agriculture, for example with the transport of pollen, spores or plant pathogens which can also be responsible for very important economic losses at a global level [11,12]; the climate, because the presence of airborne bacteria can favor the formation of clouds and precipitations by acting as condensation nuclei, potentially affecting local and global weather patterns [13]; atmospheric chemistry, by contributing to the degradation of some organic compounds [14]; ecosystems, as the bioaerosol facilitates the propagation of genetic material even over geographic barriers and long distances enabling genetic exchange between different habitats [15,16]; human and animal health by causing respiratory and allergic diseases (e.g. asthma, rhinitis, sinusitis, and hypersensitive pneumonitis), or even spread antibiotic resistance [17–19]. The SARS-CoV-2 virus is perhaps the most widely recognized example of an infectious bioaerosol, primarily due to its role in the airborne transmission of COVID-19. This has highlighted the widespread implications of airborne transmission in healthcare and public health, underscoring the critical importance of studying bioaerosols [20,21].

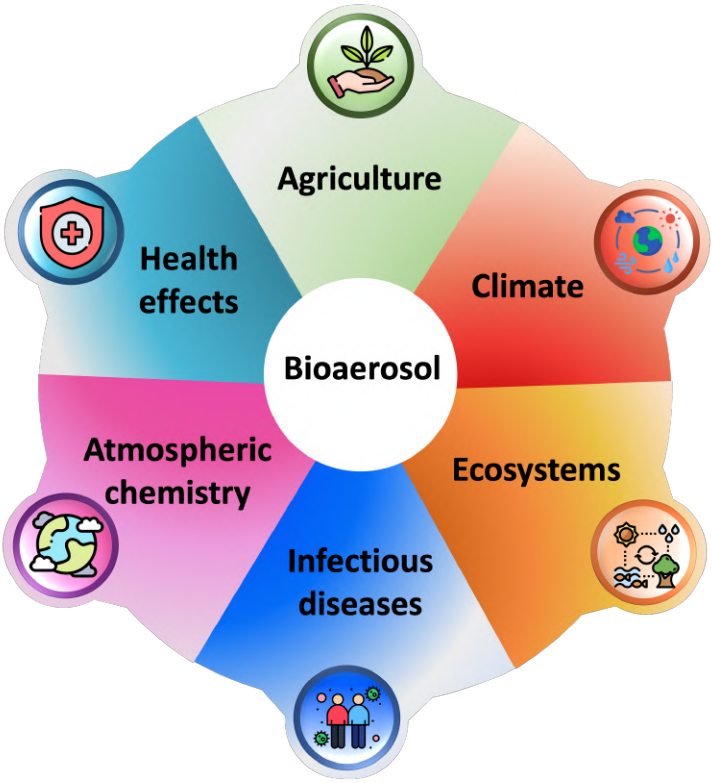

Figure 2. Impacts of bioaerosol.

Air is a very complex, variable, and diluted medium, and the bioaerosol is a dynamic component of the atmosphere. The concentration of microbial cells in the atmosphere is 10^2 to 10^6 cells/m^3 of air

[22–24]. Its fate is therefore influenced by both physical and environmental factors. Physical characteristics include the size, the density, and the shape of the particle. The environmental factors include temperature, humidity, wind, light intensity, and rain. Generally high temperatures and relative humidity (RH) favor microbiological growth, influencing the release and survival of microbial particles, such as spores, bacteria, and viruses [25]. It has been reported that long-distance transmission of airborne viruses is likely at 20 °C, as this temperature provides optimal conditions for maintaining viral stability in aerosols while reducing desiccation and degradation. For instance, these conditions enhance the ability of viruses to remain infectious over extended distances. In a field study conducted by Zhao and colleagues, it is reported that the survival of the Gumboro virus was 35-65 times higher at higher (80%) rather than lower (40%) RH conditions. These findings underline the potential for high-humidity environments to enhance airborne virus transmission, particularly over longer distances, by preserving viral integrity and preventing desiccation, as discussed earlier [22,26]. Temperature can also influence the morphological changes in dimorphic fungi, triggering shifts between mycelial and yeast states based on environmental conditions. For instance, lower temperatures often favor mycelial growth, which supports environmental survival, while higher temperatures promote yeast transformation, which is critical for pathogenicity in host organisms. In a representative study conducted by Horwath and collogues, it is demonstrated that pathogenic *Histoplasma capsulatum* may remain in a mycelial or spore form below 25 °C. These forms allow the fungus to survive in air, increasing the likelihood of human exposure and infection when conditions become favorable for transformation into the pathogenic yeast form. This transformation often enhances the pathogenic potential of certain fungi, facilitating their ability to infect host organisms and adapt to internal body conditions [27].

On the other hand, wind speed and direction play a major role in bioaerosol concentration, leading to dispersion and long-range transport. For instance, wind can carry bioaerosols from agricultural fields to distant ecosystems, spreading plant pathogens or genetic material. Lastly, rain acts by removing particles from the atmosphere through wet deposition [28].

Intuitively, bioaerosols follow seasonal patterns driven by climatic conditions and biological activity. For example, certain bacteria and viruses may show seasonal fluctuations based on temperature and humidity levels, and pollen concentrations may have a peak during spring and summer. Several studies report the highest airborne bacterial concentration during summer and fall seasons, having the lowest peak during winter [29–33]. On the other hand, high survival and transmission of some flu viruses is assessed with relatively low temperature and humidity [34].

Understanding these factors is crucial for assessing the impact of bioaerosols on ecosystems, climate change, air quality, and public health. Ongoing research remains vital to develop targeted mitigation

strategies and address the multifaceted challenges posed by bioaerosols. However, the study of bioaerosols is still in its early stages, and the evaluation of studies becomes increasingly difficult without precise sampling and quantification methods. Furthermore, the lack of standardization in bioaerosol sampling procedures is slowering the development of application studies and hampers the comparability of results obtained by different sampling devices.

1.2 Passive and active sampling

Bioaerosol sampling methods are classified into two primary categories: passive and active methods [35,36]. Despite their differences, both active and passive devices can be used in bioaerosol studies, either separately or together, depending on the specific aim of the study.

1.2.1 Passive sampling

Passive sampling is a widely used method that allows the collection of bioaerosol particles by gravity sedimentation, electrostatic force, and turbulent dispersion on a collection medium, such as a filter, agar plate, or sticky surfaces [37] (Figure 3).

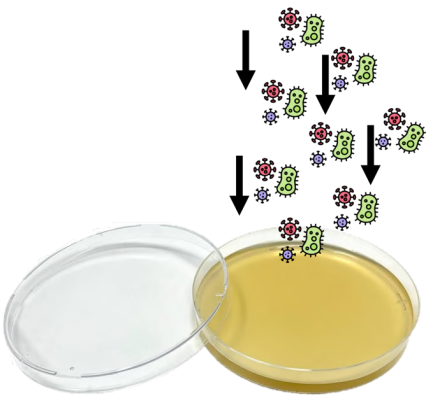

Figure 3. Bioaerosol sampling by gravity on an agar plate (passive sampling).

Unlike active sampling, this method does not actively move air, relying instead on natural forces to deposit particles onto the collection medium. The rate at which particles sediment depends on the aerodynamic properties of the particle itself, since larger particles (> 5 μm) tend to deposit faster than the small ones (< 5μm) [38,39].

Generally, passive methods make use of sedimentation plates, which are exposed in a specific environment for a set period, enabling long-term samplings, and providing information on

sedimentation rates. This is useful to study temporal bioaerosol community trends, as well as air dynamics and spatial variability in the sampling site, since different places (in the same environment) can be characterized in a given time [37,40]. Therefore, it provides more representative measure of overall exposure compared to short-duration active sampling.

Passive sampling represents a simple and cost-effective method for the collection of bioaerosol, as it does not require complicated equipment and expensive pumps or power sources. It is particularly advantageous in situations such as remote or resource-limited locations, where active sampling devices may be impractical. Moreover, its noiseless operation and minimal space requirements make it suitable for sensitive environments like hospitals or laboratories [41].

Despite the presence of many studies in outdoor sites (e.g. [42,43]), passive sampling is employed mainly to monitor bioaerosols in indoor environments such as residential buildings, schools, hospitals or workplaces to measure long-term exposure to airborne pathogens [44–47].

However, passive sampling has the major drawback to be typically considered a qualitative technique, as the collected air volume is unknown (only given exposure time is known) and small particles tend to be excluded, resulting in a misrepresentation [37]. The deposition rate of particles can be influenced by several variables, making it difficult to directly correlate the amount of bioaerosols collected with the actual air concentration [48].

1.2.2 Active sampling

Active sampling is another approach for the collection of airborne biological particles. Unlike passive sampling methods, active sampling involves the use of mechanical devices to actively draw air to a collection medium. All active bioaerosol sampling systems consist of four basic parts: the inlet, the mechanism which transports the sampled air through the device, the collection medium, and the air pump with flow monitoring. Sometimes a particle size sorter may also be present [49].

Active sampling enables the quantification of bioaerosol concentrations by measuring the total volume of sampled air, allowing for the calculation of data on particle concentrations (e.g., CFU/m³ or genome copies/m³). Furthermore, it is possible to target particle size ranges, as some samplers, such as impactors, can separate bioaerosols by aerodynamic diameter, enabling studies on size distribution and health effects. Compared to passive sampling, which is used usually for long-term samplings, active sampling may require a shorter sampling time, depending on the flow rate of the device [36].

Bioaerosol samplers may vary considerably in size and weight, from large static samplers to smaller, portable ones. Flow rates may also vary from 2-12.5 L/min to high flow rates such as 50-800 L/min, which allows collecting larger volumes of bioaerosol in a shorter time [50–53].

The collection medium is normally agar, liquid, or a filter. Liquid collection provokes less stress on airborne microorganisms, preventing desiccation and thereby maintaining viability compared to the other two sampling methods [49]. However, liquid-based samplers may experience collection losses through evaporation of the liquid medium and adhesion of the particles to the collection chamber walls.

During active sampling, if the particles are forced to pass through a tube to reach the sampler, at the same density the larger particles succumb more easily than the smaller particles due to inertial impact, hitting the wall at the bends of the tube. For this reason, the design of the inlet combined with the optimization of the air flow is essential in collecting a representative sample that correctly reflects the concentration and size distribution of the particles suspended in the air [54] (Figure 4).

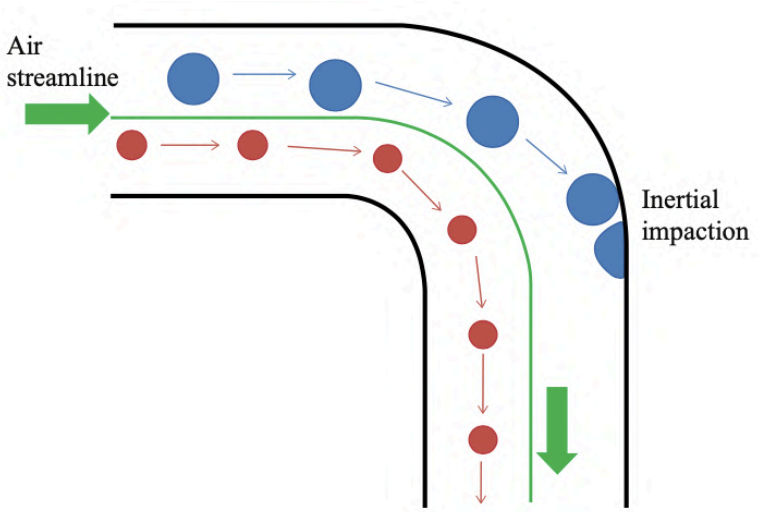

Figure 4. Inertial impaction of a pipe [46]

Other limitations of active bioaerosol sampling methods are: viability loss during collection, as the stress of sampling may also result in a loss of culturability; particle re-entrainment; bias towards larger particles, as some devices may under-sample fine bioaerosols; energy demand, as the devices require power sources (e.g., not practical for remote locations) [36].

Since there are several different air sampling system devices available on the market, considerations in the selection of a bioaerosol sampler must be conducted very accurately, based on the type and size of the microorganisms under examination, the environment in which the sampling is to be carried out, and the cost [49]. Despite this, studies have demonstrated that different results may be obtained even if different samplers are collocated in the same environment simultaneously. This once again

highlights how carefully the sampling method should be considered when interpreting the results, particularly when comparing multiple studies that use different sampling techniques [53].

1.3 Active sampling system devices

Active sampling involves the use of mechanical devices, such as pumps or vacuum systems, to draw air through a collection device from the inlet to the collection medium. The selection of the bioaerosol sampler for a specific study is crucial for assuring reliable data. Tailoring the choice of sampler to the objectives of the study ensures both accuracy and efficiency. It essential to consider factors such as the type and size of microorganisms being targeted, the sampling site, ease of cleaning and disinfection, and economic availability. Most of these factors can be revealed in the practical use in the field made of the samplers by researchers. An ideal sampler is defined by its high collection efficiency, rapid and continuous microorganism recovery in the collection medium, and ease of portability and operation [49]. However, these attributes can be influenced by factors such as wind direction, velocity, particle morphology, and inlet design [35]. Furthermore, in studies aimed to detect airborne pathogens, researchers must evaluate the *bioefficiency* of the sampler, which refers to its ability to maintain bioaerosol viability during and after sampling, ideally in laboratory conditions (since such tests are typically not provided by manufacturers) [55]. This involves spiking the sampler with a known concentration of a microorganism and analyze the collected concentration. Each microorganism reacts differently to environmental conditions and mechanical stresses, making the selection of an all-around suitable sampling device challenging.

Testing the sampler-microorganism combination in a controlled laboratory setting, ideally under humidity conditions similar to those expected in the field, is therefore crucial. The bioefficiency of the sampler must remain consistent throughout the sampling period. Factors such as environmental conditions, including humidity and temperature, can influence bioefficiency over time. For instance, high humidity may enhance microorganism survival by reducing desiccation, while low humidity can increase particle stress, lowering viability. Similarly, extreme temperatures can impact bioaerosol integrity, with cold conditions potentially preserving particles and heat causing degradation. Additionally, fluctuations in these conditions can impact sampler performance by altering airflow dynamics or causing condensation, potentially leading to inconsistent collection efficiency. Mechanical stresses during prolonged sampling or changes in airflow dynamics may affect the performance of the device used, potentially leading to biased results. Once the performance of the sampler has been validated, field bioaerosol sampling becomes more reliable [49].

Various methodologies have been developed for bioaerosol sampling; however, to date, no standardized methods have been established. This lack of standardization poses challenges for comparing data across studies, as variations in sampling techniques can lead to discrepancies in bioaerosol concentration, size distribution, and viability results. Establishing universal or even application-dedicated protocols would enhance data comparability, facilitate meta-analyses, and promote the reproducibility of findings in bioaerosol research. The principal typologies of sampling devices are described in the following sections (Figure 5, Table 1).

1.3.1 Impactors

Impactors are one of the most widely used bioaerosol sampling devices [56,57]. The air passes through a series of nozzles and it is impacted over the collecting surface, perpendicular to the nozzles. Particles with lower inertia follow the streamlines and escape, while those with greater inertia impact the agar plate due to centrifugal force. By utilizing inertial impaction principles, impactors can separate particles according to their aerodynamic diameter, which is crucial for example for studying how different sizes of bioaerosols behave in the environment and how they may affect human health when inhaled [58].

Numerous varieties of impactor devices exist, which may differ in the inlet size and shape, number of collection chambers (stages), jet-to-plate distance, and collection surface (i.e., solid like glass slide, semi-solid like agar plates, filters or gelatin) [59,60]. Examples of commercially available impactors include devices such as the Andersen multi-stage and the Aerotech N-6 (Aerotech Laboratories, Coventry, UK), which are widely used for their efficiency in separating particles by aerodynamic diameter and their versatility in diverse applications, such as public health monitoring and environmental studies. Other notable options include the Air Samplair Mas-100 (Merck, Lyon, France), the BioStage impactor (SKC, Inc.), and the Two Stage Viable Anderson Cascade Impactor (TE-10-860 TISCH Env. USA) [49,61].

In particular, the cascade impactors (multi-stages) can be used to obtain insights on the particle size distribution of the bioaerosol. The airflow passes through consecutive levels of nozzles and impact plates by centrifugal forces or inertial impaction. Each level, called stage, collects particles of a specific size while the smaller particles pass to the next stage. In fact, at each successive stage, the nozzle diameter becomes progressively smaller, increasing the air flow and decreasing the cut-off particle size. At the last stage, the airflow passes through a filter to allow the capture of the remaining particles. By weighting the plates of each stage and filters, before and after the sampling, the fraction of the total mass of particles can be established. This procedure can be useful in qualitative assessment of bioaerosols [35].

The ease of use, portability, affordability, and capacity to measure culturable populations of bacteria and fungi per volume of air are the advantages of impactors. However, these devices also have notable disadvantages, including a loss of microorganism viability due to impact stress during collection and a potential reduction in recovery efficiency caused by microorganisms failing to adhere properly to the collection surface [62].

1.3.2 Impingers

Impingers operate by directing the air flow through nozzles into a liquid medium, typically a buffer solution or water, in which the bioaerosol is sampled by impaction and diffusion. This kind of device typically relies on inertial or centrifugal forces to collect particles, regardless of the different designs it may have. The distance between the nozzle outlet and the liquid surface, along with the flow rate, influences the diameter of collected particles [49,63]. The liquid serves both as collection medium and as preservative, since it prevents the drying of the sampled bioaerosol particles resulting in a higher bioefficiency. Therefore, impingers are usually used in studies focused on potential airborne pathogens in which it is mandatory to collect and enumerate viable microorganisms. However, shear forces along with turbulence caused by the air flow can result in a partial loss of viability of the microorganisms. The bioefficiency can also be compromised in part by evaporation, the re-aerosolization of particles, and the adhesion of particles to the internal walls of the collection chamber [64,65].

The most commonly used impingers for bioaerosol samplings are the All-Glass Impinger (Ace Glass Inc., Vineland, NJ, USA), the BioSampler (SKC, Eighty Four, PA, USA), the Multistage Liquid Impinger (Burkard Manufacturing Co. Ltd, Rickmansworth, UK), and the Coriolis Micro - Microbial air sampler (Bertin Technologies France) [49,66].

1.3.3 Cyclones

The underlying principle of cyclone samplers relies on centrifugal force, where the air is directed by a swirling flow following the shape of the collection chamber into the liquid medium [49]. Although collecting particles on a liquid medium maintains the viability of the bioaerosol, shear forces can still have an effect on the vitality of microorganisms, as well as the evaporation of the collection medium [67]. Recently companies focused on the development of automated wet-cyclone systems for rapid, continuous, and enriched bioaerosols sampling. An example of such a system is the Coriolis® RECON (Bertin Technologies, France), which offers high-efficiency collection and real-time monitoring capabilities, making it suitable for applications in environmental and public health surveillance [68]. The high flow rate ratio between the incoming air and drainage liquid allows these

systems to achieve great sampling performances and permits sample concentration. To increase the bioefficiency, a small amount of liquid is injected near the inlet of the cyclone, in order to wet the walls of the device. Thus, wet-cyclone samplers can reduce the desiccation, particle bounce, impaction stress, and re-entrainment thus substantially improving the biological collection efficiencies [69,70].

Cyclones may vary in size and flow rate (up to 1250 L/min), which influence the collection efficiency [71].

1.3.4 Filtrators

Filtration based samplers have a relatively simple mechanism, thus it is the most common system used for bioaerosol sampling. Bioaerosol is collected by passing through porous membrane filters, which may be made of glass fibers, polyvinylchloride, polycarbonate, cellulose acetate, Teflon or gelatin by producing vacuum by the use of a pump [72,73]. A recent study evaluated three types of filters (i.e., polycarbonate, gelatin, and glass fiber) for bacterial studies using liquid culture and DNA quantification. The results indicated that polycarbonate filters outperformed the other two. Similarly, another study reported that polycarbonate filters were superior to gelatin and polytetrafluoroethylene filters for fungal recovery, as demonstrated through enumeration and advanced monitoring techniques. Moreover, polycarbonate filters were found to be the most effective for bacterial recovery as well [73].

As particle-laden air enters the membrane filter, the particles settle on the pore structures. The filter is typically selected based on the size range of particles to be collected, which can range from large fungal spores to very fine particles such as bacteria or viruses [35].

Air particles are sampled by inertial forces, diffusion and electrostatic attraction [74]. After collection, the filter can be analyzed using various techniques, including culture-based methods, molecular diagnostics, or microscopy, to identify and quantify the microorganisms present in the sample [75].

Filtration is highly efficient at sampling bioaerosols, particularly in comparison to other sampling techniques such as impingers or impactors, which may not always collect efficiently fine particles. However, when the sampling site is a highly contaminated environment, overloading of filters may occur reducing the ability to sample additional bioaerosol (under-sampling). Furthermore, desiccation of the sample post collection is another problem related to filtration based samplers [75,76].

Two examples of filtration-based samplers are the Airport MD8 (Sartorius, Goettingen, Germany) and the Bobcat Air Sampler (Innovaprep). The Airport MD8 uses an 80 mm diameter gelatin membrane filter (3 μm pore size) that fits directly into the unit without the use of tubing [77]. The Bobcat Air Sampler uses electret filters made from a combination of positively and negatively

charged fibers that can intercept viruses and bacteria, which are then eluted using a rapid elution kit [78]. Electret filters and related technologies worth attention for future experimental intercomparison studies.

1.3.5 Growth tube collectors

Bioaerosol sampling using condensation techniques involves passing the air sample through porous tubes conditioned by water and set at different temperatures; namely a humidifier where warm, moist air is then rapidly cooled with the bioaerosol particles acting as condensation nuclei [49]. The cold and wet wall in the conditioner elevates the RH of the airflow to nearly 100%. Upon entering the initiator, the hot and wet wall rapidly increases the temperature of the cold, humidified flow, resulting in supersaturation and subsequent water droplet formation on the surface of the particles [79]. This process enhances the size of the particles, facilitating their capture. By increasing particle size through water condensation, the physical collection efficiency for airborne microorganisms having a small diameter (i.e., viruses) is significantly improved. Furthermore, the water enveloping the particles minimizes the impact during collection, ensuring the preservation of a substantial number of viable microorganisms [80]. In a study conducted by Silva et al., it was shown that collecting viral aerosols using condensation is much more efficient than sampling by impingement in terms of bioefficiency [81].

Although this method can be used effectively to detect microorganisms in the bioaerosol, the system is complex to operate and heat transfer to the microbes can result in a partial loss of viability [49]. Furthermore, conventional growth tube collectors operate at significantly lower flow rates, typically around 1-8 liters per minute (LPM), compared to the much higher flow rates of commercially available air samplers, which range from several tens to several hundreds of LPM. Standard growth tube collectors are commonly composed of eight sampling tubes, each with a diameter of 9 mm, a flow rate of approximately 0.875 LPM, and an average flow velocity of 0.23 m/s [79,80,82].

One of the growth tube collectors available on the market is the BioSpot-ViVAS (Aerosol Devices Inc., Ft. Collins, CO, USA).

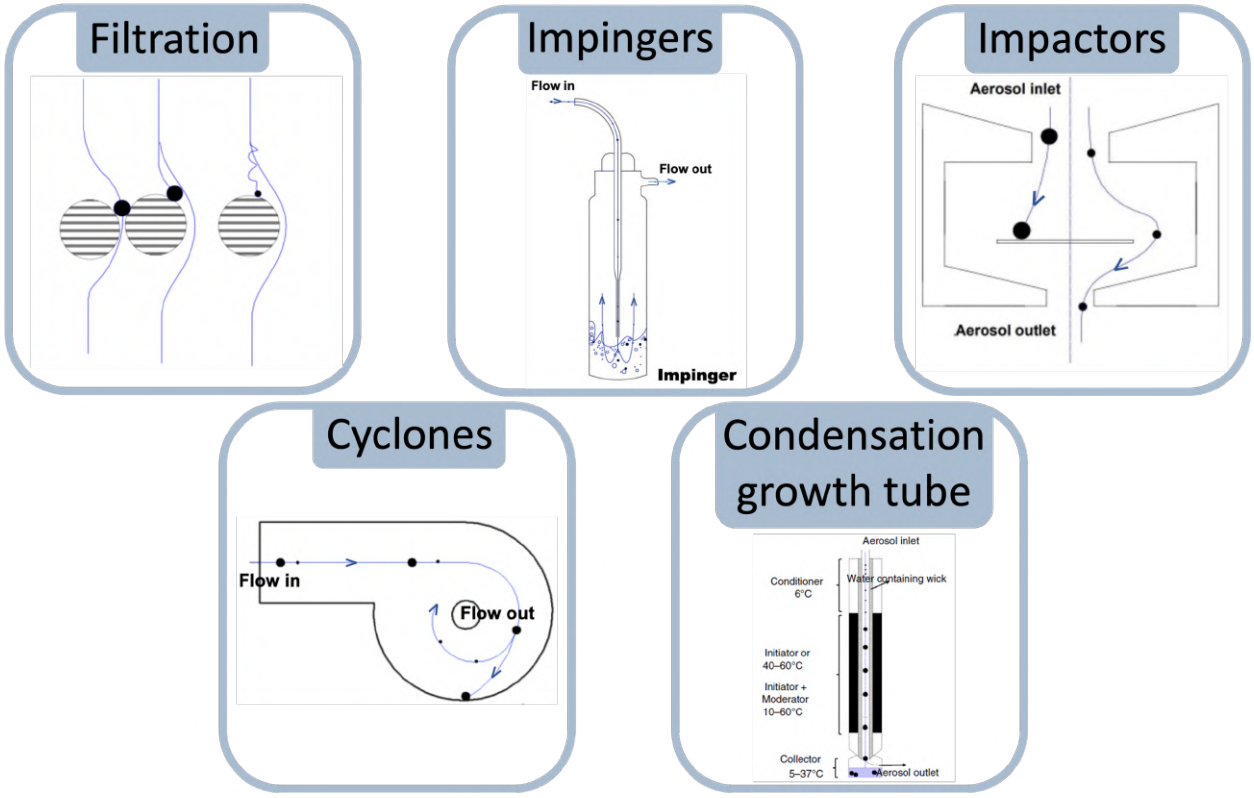

Figure 5. Primary sampling systems for bioaerosol collection.

Table 1. Advantages and disadvantages of the main bioaerosol sampling techniques [32].

Active/passive sampling	Bioaerosol sampling technique	Advantages	Disadvantages
Active sampling	Impaction	-Widely used due to economic feasibility. -Direct collection of microorganisms on to growth medium reduces the post sampling process required. -Multiple samples can be processed without sterilizing the sampler in between the sampling. -Inhalable fractions of bioaerosols are sampled by several commercially	-When sampled in highly contaminated site culture plates get overloaded making enumeration difficult due to overlap of colonies.

		available impaction samplers.	
Active sampling	Impingement	-Technique widely used hence considerable amount of data on collection and efficiencies are available. -Use of liquid collection medium instead of solid reduces the problem of overloading as well as loss due to physical stress on microorganisms. -No restriction on the type of enumeration technique used subsequently.	-Post collection processes required for quantification. -Sterilization of the sampler is required between consequent samplings. -Due to evaporation of liquid medium problem of loss may be encountered. speed during sampling. -Liquid sampling is not compatible with size fractionation.
Active sampling	Cyclone	- Good collection efficiency because of reduced particle bounce and loss through re-entrainment. -Easy sterilization process.	-Due to evaporation of liquid medium problem of loss may be encountered.
Active sampling	Filtration	-Simple and economically feasible. -No restriction on the type of enumeration technique used subsequently. -Includes the potential for size fractionation.	-Post collection processes required for quantification. -Filters are prone to overloading when sampling in highly contaminated environments. -Low recovery efficiency due to

			desiccation of microbes on filters.
Active sampling	Growth tube collection	-Ultrafine bioaerosol particles can also be sampled and detected easily. -Viability of the microorganisms maintained throughout.	-Complex system requiring expertise to handle. -Low flow rate.
Passive sampling	Gravity	-Easily available and economically feasible. -Many samples can be taken from different places at the same time. -Comparable and reliable results. -Reproduce real conditions.	-Greatly relies on air currents. -Bias towards larger particles. -Weakly correlated with counts of other quantitative methods. -Weakly correlated with defined volume of surrounding air. -Long sampling time compared to other techniques.

1.4 Analysis of bioaerosol

Literature reports variable bioaerosol concentration ranges, depending on considered microorganisms [35]. For instance, bacterial bioaeosols range from 10^2 to 10^6 CFU/m^3 [83–85], while studies on virus concentrations are limited due to technical challenges. Griffin and colleagues collected airborne viruses and bacteria in African desert dust and found that concentrations were similar for the two types of bioaerosols, ranging from 10^4 to 10^5 particles/m^3 [86]. In another study conducted by Prussin and colleagues on the concentrations of total bacteria and viruses in indoor air, virus-like particle concentrations of approximately 10^5 particles/m^3 and bacteria-like particle concentrations of

approximately 10^6 particles/m^3 were reported in various indoor environments and outdoor air, respectively [87].

The low-biomass condition of microorganisms in the atmosphere may complicate the subsequent analysis and quantification of the sampled bioaerosol, as it can lead to difficulties in detecting or amplifying target organisms due to their limited representation in the sample. This scarcity can result in insufficient material for downstream molecular or culture-based assays, reducing the reliability of results. Rigorous cleaning and sterilization of samplers and laboratory workspaces are essential to prevent bias and contaminations. Experimental blanks should be routinely compared to verify the accuracy of results, and repeated subculturing must be performed to ensure consistency and eliminate potential contaminants.

Bioaerosol analysis has been divided into two main methodologies: the culture-dependent techniques, that require a culture medium in which the microorganisms grow, and the culture-independent techniques that do not require a culture medium but make use of molecular methods for the direct identification of the microorganisms. The main difference between the two methods is that in the first case, all the "non-viable" and "viable but not culturable" species are excluded from the analysis. Instead, in the second case, all the species (including the "non-viable" and "non-culturable" microorganisms) can be identified.

1.4.1 Culture-dependent techniques

From its beginning, microbiology has relied on culture methods to identify microorganisms [88]. In fact, prior to the development of molecular and advanced physicochemical techniques, the diversity, identity, and concentration of airborne microorganisms were mainly investigated through microscopic examination and cultivation on selective and non-selective media. These approaches have a long-standing tradition in aerobiological research and still continue to be valuable and cost-effective tools for the bioaerosol analysis [72].

The culture-based approach involves the collection of viable airborne microorganisms in a liquid or semisolid growth medium. After a proper incubation (set time, temperature, and available oxygen), assuming that a single colony is generated from a single microorganism, the results are expressed in colony forming units (CFU), referring to the number of microorganisms present in that sample [49,89]. Colonies can then be enumerated, subcultured, and further characterized to determine the identity, diversity, and concentration of the sampled bioaerosol through morphological examination, biochemical tests, or subsequent molecular techniques.

Culture-dependent techniques are useful when the interest lies in the viable fraction of bioaerosol, for species that respond well to the culturing method used. In fact, only the viable microorganisms will grow on the medium, and this can lead to an underestimation of the microbial diversity and concentration of the sampled environment [90]. Furthermore, some microorganisms might not grow on the medium even if they are viable. It is well known that the vast majority of environmental microbiota is non-culturable, even when viable [4,91–94], e.g., ~ 1% of bacteria [95] and ~ 17% of fungi [96].

The full range of atmospheric microbial diversity is now partially becoming accessible thanks to recent advancements and applications of DNA and RNA based techniques [72].

1.4.2 eDNA and culture-independent molecular techniques

Traditional culture-based methods, while informative, are limited by their inability to detect non-culturable or rare microorganisms, which often constitute a significant portion of airborne microbial communities [97]. In recent years, culture-independent analyses have been applied to various areas of airborne microbiology research to assess microbial diversity, increasing the specificity and sensitivity of environmental studies [98–101]. The entire atmospheric microbial diversity is now becoming accessible through recent developments and applications of DNA- and RNA-based methods [94,102]. In particular, the advent of high-throughput sequencing technologies (Next Generation Sequencing technologies, NGS) has revolutionized the microbial ecology, enabling comprehensive analyses of complex microbial communities.

The environmental DNA or RNA (eDNA or eRNA) method is a non-invasive approach to detect and identify species from cells or extracellular DNA/RNA present in environmental samples (e.g., air, water, soil). For instance, this method has been particularly effective in monitoring biodiversity in aquatic ecosystems, tracking invasive species in freshwater habitats, and studying microbial dynamics in soil environments [103,104]. This is achieved through techniques such as DNA metabarcoding or metagenomics (DNA)/transcriptomics (RNA), which enable the characterization of microbial communities from complex environmental matrices [105].

DNA metabarcoding

Metabarcoding has become a pivotal technique in microbial ecology, offering a high-throughput approach to study the diversity and composition of microbial communities in a wide array of environments. This technique involves the amplification and sequencing of taxonomically informative genetic markers (called “DNA barcodes”), such as ribosomal RNA genes or other short DNA sequences, allowing for the simultaneous identification of many taxa within the same sample.

An effective DNA barcode should exhibit minimal variation within species (intraspecific) while demonstrating significant variation between species (interspecific). It is characterized by strong evolutionary conservation, allowing the use of a combination of conserved universal Polymerase Chain Reaction (PCR) primers flanking a short hypervariable region. This specific region is amplified in a thermocycler, where the DNA region of a genome is copied and amplified a million-fold in less than an hour, making it suitable for further analysis. Its ability to provide taxonomic resolution across diverse microbial taxa makes it a powerful tool for profiling microbiomes in a variety of matrices, including bioaerosols (Figure 6).

Different barcodes are used to identify different groups of organisms because the genetic markers vary in their suitability for distinguishing different taxa. For instance, prokaryotes (bacteria and archea) are best identified using the 16S rRNA gene as a barcode, while fungi require the ITS (Internal Trascribed Spacer) region, and microbial prokaryotes the 18S rRNA gene. Reference libraries are then used for the taxonomic identification (annotation) of sequences obtained from metabarcoding. These databases contain the DNA barcode assigned to previously identified taxa [106].

The workflow of the metabarcoding technique involves several key steps. First, samples are collected, and the isolation of the DNA is performed. Next, specific hypervariable regions are amplified (such as the V3-V4 or V4-V5 regions of the 16S rRNA for bacterial identification) through PCR using universal primers. Finally, the amplified regions are sequenced using high-throughput platforms such as Illumina MiSeq or NextSeq. The resulting millions of reads are processed through bioinformatics pipelines to identify microbial taxonomic composition and assess relative abundances [107].

The strength of 16S rRNA metabarcoding lies in its ability to detect both abundant and rare taxa, enabling a detailed characterization of microbial diversity within bioaerosols. Furthermore, this approach allows the identification of potentially pathogenic taxa, providing valuable insights into public health implications, particularly in occupational settings or urban environments [108,109].

While the metabarcoding technique offers significant advantages in detecting microbial diversity and identifying pathogenic taxa, its application to bioaerosol research is not without limitations. In fact, bioaerosols present unique challenges for characterization due to their low biomass, dynamic nature, and diverse composition. The low microbial biomass necessitates sensitive and precise analytical techniques, as insufficient DNA or RNA quantities can compromise detection.

The reliance of the metabarcoding technique on the PCR, which is a critical step in the workflow, may introduce potential biases. First, non-specific product amplification may be generated. Secondly, preferential amplification and the formation of chimeric sequences can distort community composition estimates [110,111]. Another important point that must be considered is the possible presence of PCR inhibitors, which ties closely to challenges in DNA extraction and amplification

processes. Addressing these inhibitors is critical to ensure accurate and reliable results. Substances such as humic acids inhibit the DNA amplification process either by hindering the attachment of polymerase to the primers, or by binding to the DNA and thereby preventing primers or enzymes from attaching. Different PCR primers as well as DNA polymerases vary in their ability to overcome inhibitory factors. In addition, filter materials on which biological aerosol particles are collected can also inhibit the PCR amplification [97].

Although DNA is a stable molecule, factors such as UV light, ozone, and melting-freezing cycles accelerate its degradation. This leads to the deterioration of DNA and the eventual loss of genetic information (e.g., when biological material remains airborne for extended periods). Researchers often mitigate this issue by promptly collecting samples and employing preservation methods such as storing filters at low temperatures or using stabilizing agents to protect genetic material until analysis. Pollen grains, as well as many spores of fungi and bacteria, are encased by thick cell walls to protect their DNA from DNA-destroying environmental processes, while for example viruses are more sensitive [112].

The resolution of metabarcoding data is also constrained by the quality of reference databases, which may lack of comprehensive or accurate representations of airborne microbial taxa. Furthermore, the absence of standardized protocols for bioaerosol sampling, DNA extraction, and data analysis complicates the comparison of results across studies [111].

Another drawback of the metabarcoding technique is attributed to the need to analyze different groups of organisms (archaea, bacteria, fungi, plants, viruses, etc.) separately. This requirement increases the time and resource demands of the workflow, as distinct protocols and analyses must be conducted for each group, complicating comprehensive assessments of microbial diversity. As a consequence, it is extremely difficult to assess the relative proportion of each group within a DNA extract [106].

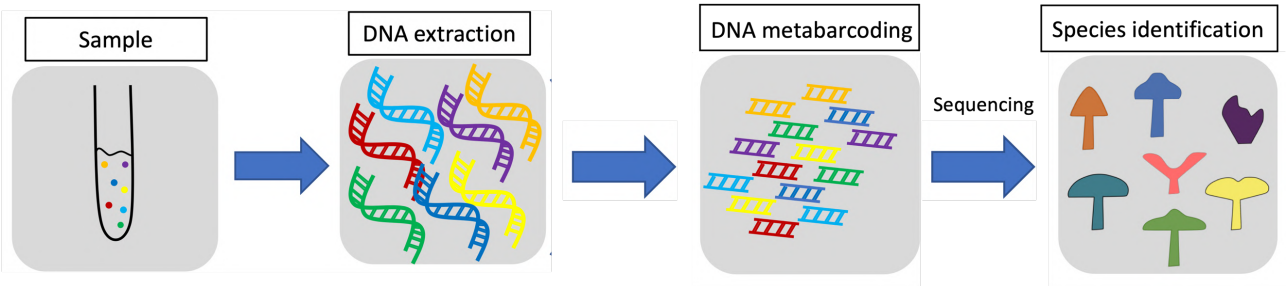

Figure 6. Workflow of the DNA metabarcoding approach.

Metagenomics

While in the metabarcoding approach, a gene or a few genes are sequenced and used primarily to carry out phylogenetic-type studies, in the shotgun metagenomics, all the present DNA in a sample

is sequenced and used in functional gene analysis assays [113]. DNA is isolated from environmental samples and broken into small fragments that are then sequenced on NGS technologies such as Illumina HiSeq or PacBio. The fragments are then assembled and advanced computational tools are employed to process and analyze the data, including quality control, assembly, annotation, and taxonomic and functional profiling [114] (Figure 7).

As previously said, metabarcoding studies rely on PCR amplification of hypervariable regions of DNA to identify specific groups of species (e.g., bacteria). In contrast, shotgun metagenomics involves isolating DNA from samples and directly sequencing the entire genetic material. This provides a comprehensive analysis of the taxonomic and functional microbial diversity and composition of the whole microorganism population present in an environmental (aerosol) sample [115,116]. Therefore, this approach has provided a much deeper understanding of relationships between different elements of the genome, allowing to draw functional conclusions on specific genes (e.g., assessing antibiotic resistance genes) [117].

However, metagenomics requires a large quantity of genetic material to be collected during environmental sampling to have a sufficient amount of nucleic acid for the sequencing process [118]. This entails collecting a huge volume of air per sample, which appears challenging for most of the air sampling devices, which are characterized by a low flow rate. Improving the collection efficiency of air sampling devices would help mitigate this [60]. To address the challenge of low DNA yield from air samples, some metagenomic protocols include an additional step to amplify environmental DNA prior to sequencing, employing techniques such as Multiple Displacement Amplification. This method can generate micrograms of DNA from femtograms of starting material, ensuring adequate quantities of DNA for successful sequencing. However, amplification processes can introduce primer mismatches, chimeric sequences, and biases, whose severity depends on the quantity and quality of the initial genetic material as well as the number of amplification cycles performed [119,120]. Another approach to increase the biomass of the sample involves culturing microorganisms obtained through active sampling before proceeding to genetic analyses. However, it inherently introduces biases in the representativeness of the sample, as it favors microorganisms that are culturable under laboratory conditions (as mentioned earlier, see Culture-dependent techniques) [60].

Despite the challenges associated with analyzing bioaerosol samples using the metagenomic shotgun approach, several recent studies have demonstrated its feasibility and effectiveness [83,121–123].

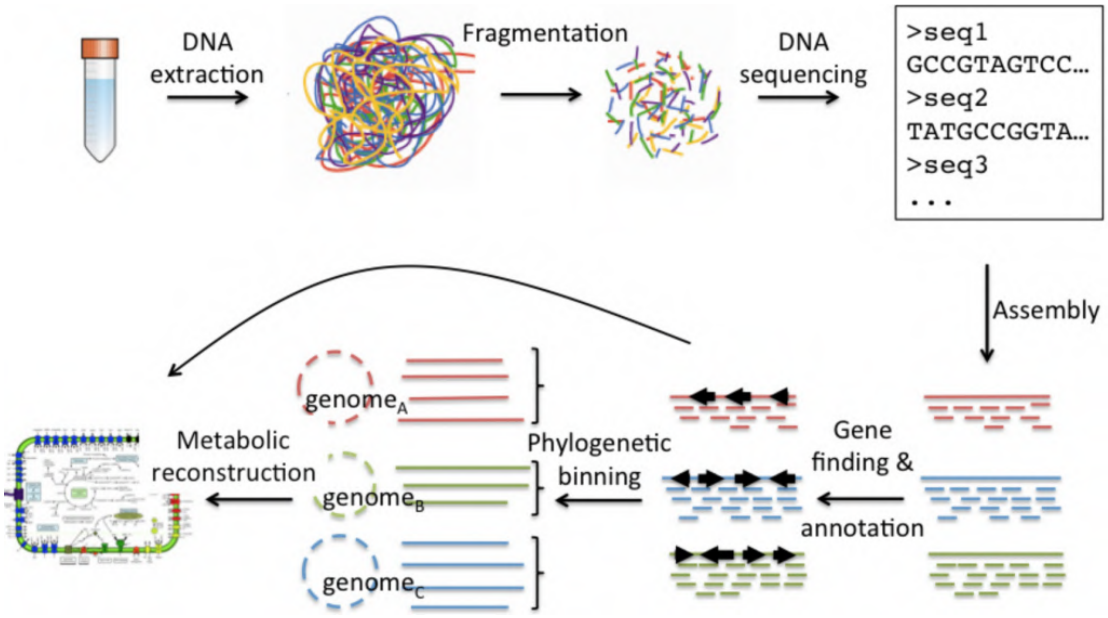

Figure 7. Workflow of the shotgun metagenomic approach.

2 Aim and rationale of the thesis

The presence and influence of bioaerosol across various environments presents substantial risks to public health, agriculture, and bio-threat surveillance. However, the study of bioaerosols is still in its infancy, and variability in natural and anthropogenic sources, coupled with the lack of standardized sampling methodologies, often results in inconsistent and non-comparable findings. Despite advancements in both culture-dependent and culture-independent methods, accurately detecting microorganisms (in particular pathogens are of high concern) in the complex and dynamic atmosphere remains a significant obstacle. These limitations hinder the development of definitive conclusions and underscore the need for further research to improve our understanding and management of bioaerosol-related risks.

It is clear that each method used to study bioaerosols has its own limitations, hence combining different technologies can provide a comprehensive solution to overcome any drawbacks. For instance, integrating passive and active sampling methods can provide both long-term exposure data and precise quantification of bioaerosols. Similarly, coupling culture-dependent and culture-independent techniques allows researchers to capture a more complete picture of microbial diversity, addressing both viable and non-viable fractions. For this reason, omics approaches provide a promising method for air microbiology study. In particular, making use of high-throughput sequencing technologies allows to achieve detailed and relevant insights into metabolic capacity,

activity, and interactions at the DNA, RNA, and protein levels. By applying these techniques, researchers can address key gaps in bioaerosol research, such as identifying previously uncharacterized microorganisms and understanding their ecological roles and potential health impacts.

Overcoming these challenges is crucial for enabling accurate assessments and support the development of effective strategies to mitigate the risks posed by bioaerosols in diverse environments. Additionally, emerging threats like COVID-19 have underscored the critical importance of developing more efficient and rapid bioaerosol detection methods, particularly in healthcare settings.

The primary aim of this thesis is to advance the understanding of bioaerosol sampling methodologies in both indoor and outdoor environments, with a focus on their applicability in diverse environmental contexts and their role in assessing airborne contamination and public health impacts. This broad objective involves refining sampling designs and techniques, as well as improving the subsequent laboratory analysis through the development of innovative protocols, thereby enabling the characterization of specific indoor settings such as healthcare facilities, as well as outdoor environments like wastewater treatment plants. Furthermore, this thesis seeks to study bioaerosols within controlled settings, such as specialized laboratory chambers simulating varying humidity, concentrations of microorganisms, and airflow conditions, by conducting targeted experiments to elucidate the underlying dynamics governing bioaerosol behavior. A further goal is to evaluate mitigation and sanitation technologies. This involves assessing their effectiveness under various conditions and contributing to the development of novel operational protocols and experimental frameworks.

After the general introduction on bioaerosols, the thesis is organized in five sections and a concluding resume. The first two sections report published results describing the development – in laboratory setting – of original setups for the assessment of filtration devices (e.g., personal nasal filters coated by silver nanoparticles, and a hyper-reflective UV-C LED aerodynamic sintered PTFE filter module), that have been designed and assembled for the testing of two typologies of filters, by aerosolization, transmission, sampling, and count of *E. coli* as model organism. The further three sections move to indoor and outdoor applications of bioaerosol characterization in real world scenarios. The third section reports an unpublished research that deals with the characterization of the spread of airborne culturable and settling bioaerosol in a hospital ward recognized for high bioaerosol production, namely the dentistry ward, with a case study conducted at the Maggiore Hospital in the city of Trieste (Italy). The fourth and fifth sections report studies conducted at a type of technological plant where

bioaerosols are constantly formed: the waste water treatment plant (WWTP). In particular, the fourth section reports a published research on an experimental comparison among different air samplers operating in close proximity at the aeration tank of the WWTP of Trieste (Italy), with collected material analyzed using the 16S rRNA metabarcoding technique. The fifth section describe an unpublished study aimed at identifying broad spectrum antimicrobic resistant bacteria by high performing molecular technologies as metagenomics (involving Next Generation Sequencing platform). The bioaerosol samplings did not yield sufficient genetic material for a metagenomic study. Therefore, the focus has been directed to the characterization of antibiotic pressure in wastewaters as a source for antimicrobial resistance genes and bacterial community that can be aerosolized.

Conclusions resume overall results and highlight issues that constitute support to innovation in the field of bioaerosol characterization and sanitation science and technology.

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SECTION 1

Operative protocol for testing the effectiveness of Nasal Filters in Preventing Airborne Transmission of SARS-CoV-2

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Published on International Journal of *Environmental Research and Public Health*, https://doi.org/10.3390/ijerph192113790

Abstract

Background: Standardized methods for testing Viral Filtration Efficiency (VFE) of tissues and devices are lacking and few studies are available on aerosolizing, sampling and assessing infectivity of SARS-CoV-2 in controlled laboratory settings. NanoAg-coated endonasal filters appear a promising aid for lowering viable virus inhalation in both adult and younger populations (e.g., adolescents). Objective: to provide an adequate method for testing SARS-CoV-2 bioaerosol VFE of bio-gel Ag nanoparticles endonasal filters, by a model system, assessing residual infectivity as cytopathic effect and viral proliferation on in vitro cell cultures. Methods: A SARS-CoV-2 aerosol transmission chamber fed by a BLAM aerosol generator produces challenges (from very high viral loads (105 PFU/mL) to lower ones) for endonasal filters positioned in a Y shape sampling port connected to a Biosampler. An aerosol generator, chamber and sampler are contained in a class II cabinet in a BSL3 facility. Residual infectivity is assessed from aliquots of liquid collecting bioaerosol, sampled without and with endonasal filters. Cytopathic effect as plaque formation and viral proliferation assessed by qRT-PCR on Vero E6 cells are determined up to 7 days post inoculum. Results: Each experimental setting is replicated three times and basic statistics are calculated. Efficiency of aerosolization is determined as difference between viral load in the nebulizer and in the Biosampler at the first day of experiment. Efficiency of virus filtration is calculated as RNA viral load ratio in collected bioaerosol with and without endonasal filters at the day of the experiment. Presence of infectious virus is assessed by plaque forming unit assay and RNA viral load variations. Conclusions: A procedure and apparatus for assessing SARS-CoV-2 VFE for endonasal filters is proposed. The apparatus can be implemented for more sophisticated studies on contaminated aerosols.

1 Introduction

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus type 2) is the etiological agent of the respiratory COronaVIrus Disease 2019 (COVID-19) pandemic that from the beginning of the 2020 has spread worldwide, evolving into several variants of concern (VOCs) that have troubled the human population and the public health sector. The current more frequent variant is the Omicron (B.1.1.529), with BA.1, BA.2, BA.3, BA.4 and BA.5 as the designated descendant variants. Despite the higher transmissibility of Omicron SARS-CoV-2 BA-1 and BA.2 with respect to the previous variants, a decrease in disease severity is detected in the individuals infected [1]. Actually, BA.2, BA.4 and BA.5 are classified as variants of concern (VOCs) by the European Centre for Disease Prevention and Control (ECDC), BA.5 being the predominant sub-strain circulating in the UE; nevertheless, ECDC does not indicate an effect on severity and transmissibility, although the number of cases is continuously increasing [2]. Nevertheless, a negative impact on vaccine efficacy is regrettably denoted for these SARS-CoV-2 Omicron sub variants [3]. Moreover, a variant has evolved from variant under monitoring (VUM) to variant of interest (VOI), i.e., omicron BA.2.75. That being said, it arises that SARS-CoV-2 develops quite rapidly and the control of future strains should be mandatory to contain the pandemic, since it cannot be excluded that new variants might be more transmissible and produce more severe disease. Despite the increased transmissibility, in vitro studies have shown that the Omicron strain seems to replicate more slowly than Delta [4] and induces less syncytia formation [5]. Interestingly, Omicron spike protein (structural study [6]) and Omicron virions (functional study [7]) are more stable with respect to previous strains. It has also been estimated in a computer modeling study [8] that Omicron variant airborne transmission contributed to 34–38% of asymptomatic-pre-symptomatic phase diagnose, with respect to a negligible contribution (less than 10%) to the symptomatic COVID-19 phase, when droplet transmission is predominant (asymptomatic-pre-symptomatic stage: 21–28%; symptomatic stage: 48–71%); finally, the contact role is similar in the two disease conditions (asymptomatic-pre-symptomatic stage: 37–45%; symptomatic stage: 25–42%). This difference could be due to the size of particle emitted; indeed, in asymptomatic-pre-symptomatic infection, the virus was spread through breathing and talking (droplets < 10 μm), while during the symptomatic disease, coughing and sneezing predominates, characterized by larger dimensions that tend to deposit on surfaces. Studies of both indoor and outdoor air viral contamination require definition of protocols regarding sampling criteria [9], detailing instruments, duration, positioning and eventual sample storage [10].

Despite SARS-CoV-2 being a respiratory virus, studies on its aerosol transmission in controlled laboratory settings are still limited and standardized national or international viral filtration efficiency

testing protocols are still missing [11,12]. Studies have mainly used bacteriophage and influenza viruses or inactivated viruses as challenges in filtration tests [11,12,13,14,15]. Actually, the indications to prevent transmission among the population include social distancing, the avoiding of people-gathering situations, ventilation of the indoor environment and personal and environmental hygienic measures [16]. Not in all real-world situations can these measures be easily afforded; as an example, schools are indoor environments at high risk due to the long-term gathering of children and adolescents [17]. ECDC recommended the promotion of physical distancing through cohorting of groups and classes (on the base of infection risk and health status), distance within the classroom (e.g., spacing desks), reduction of class size, staggering the start and the end of the lessons and break times, or holding lessons in outdoor environment. Nevertheless, some of these indications are not feasible in all schools and situations [18].

Last but not least, Personal Protective Equipment (PPE), such as medical masks and respirators, have been and still represent a key feature in limiting the spread of SARS-CoV-2.

With the employment of PPE, it is possible to reduce the penetration of pathogens through the reduction of the "microbial load" in inhaled air, so decreasing the risk of transmission of many respiratory infectious agents in occupational or community settings. Moreover, the constituents of indoor air that may be involved in the exacerbation or in the induction of respiratory infections can be trapped. Filtering face respirators, hygienic and surgical masks designed as PPE, can decrease the inhaled microbial load through a simple filtration process. On the other hand, these devices trap microorganisms at the expense of a reduction in the inhaled airflow, so impacting negatively on the respiratory capacity [19]. Compared to currently available PPEs, endonasal filters [20] with silver nanoparticles (AgNPs) possess some advantages, due to the combination of the antibacterial and antiviral action of AgNPs [21] with common filtration processes. This dual mechanism reduces microbial infectivity and is able to protect the lower airways. They are expected to be used when nose airways are clean and without symptoms of rhinitis. Biogel-AgNP nasal filters can trap and inactivate bacteria and viruses, so they can be considered a promising type of PPE to be employed both in occupational and community settings [22]. In the healthcare environment, bio-gel-AgNP nasal filters may be used to prevent the transmission of microbial agents between patients and healthcare personnel, but their use can be extended to other occupational settings such as schools, universities, offices, or where a possible contamination by biological agents and/or risk of microbial transmission to humans exist. Furthermore, in the general population (both adult and younger, e.g., adolescents), bio-gel-AgNP nasal filters can contribute to the inhibition of microbial agents# transmission through air in domestic and outdoor environments, as well as when masks are not worn, e.g., break times and lunchtime at school, workplace canteens. Biogel-AgNP nasal filters can thus contribute to the

prevention of air-transmitted infectious diseases, guaranteeing long time continuous use combined with breathing comfort, for day long protection in both adult and younger populations. In order to test the efficacy of bio-gel-AgNP nasal filters in blocking SARS-CoV-2 transmission, in the following an experimental protocol for assessing SARS-CoV-2 infectivity reduction by endonasal filters will be described, where SARS-CoV-2 is aerosolized in a controlled setting inside a class II cabinet hood in a Biosafety level 3 facility.

2 Materials and methods

2.1 Rationale and specific aims

The present protocol evolves from our experimental model of SARS-CoV-2 aerosol generation and transmission employing a BLAM bioaerosol generator, a cylindrical chamber for aerosol travel and a swirling bioaerosol collector SKC Biosampler for the collection of the particles [23], and from the experimental model for assessing the SARS-CoV-2 filtering efficacy of face masks published during 2020 by Ueki and coworkers [24]. An 8-Jet BLAM nebulizer operated as Multi Pass Atomizer mimics the spread of virus particles from a COVID-19 positive emitter patient (8 L per minute) while the SKC Biosampler sucks air at flow comparable to that of human breath inspiration (12.5 L per minute). SARS-CoV-2 is aerosolized into a plexiglass parallelepiped box (100 × 40 × 50 cm) positioned in a class II cabinet hood in a Biosafety level 3 laboratory and collected through a Y shaped tube sampling port—simulating nostrils—connected to the SKC sampler. Such a sampling port is a simple model for testing the mechanical filtration performance of AgNP coated endonasal filters, as well as reduction of microbial infectivity by flow interaction with internal coated filter surfaces. The sampling setting can be modified for testing facial masks by inserting a human-like head within the bioaerosol chamber, as in [24]. Infective virus concentration is determined by use of a plaque assay and the RNA viral load quantified by employing quantitative real-time reverse transcription PCR (qRT-PCR). The efficiency of the bio-aerosol nebulization and transmission system is assessed after the aerosolization determining the viral load in the SKC sampler compared to that remaining in the nebulizer. Effectiveness of Biogel silver nanoparticle (AgNP) endonasal filters inserted into the Y shaped tube sampling port, connected to the SKC sampler simulating nose inhalation, is evaluated, measuring infective viruses that pass through the endonasal filters and are collected in the SKC bio-sampler reservoir, by use of a plaque forming unit (PFU) assay and quantitative reverse transcription real-time PCR (qRT-PCR) [23].

2.2 Experimental setting and procedure

A model for SARS-CoV-2 air contamination, airborne transmission and viral filtration efficiency testing is constituted by a plexiglass test chamber (100-cm long × 40-cm wide × 50-cm high - Figure 8) with removable circular plexiglass closures, holed to allow bioaerosol input (from BLAM bioaerosol generator) and collection (by SKC Biosampler) in the opposite small sides of the test chamber. This device represents the first containment for viral bio-aerosol.

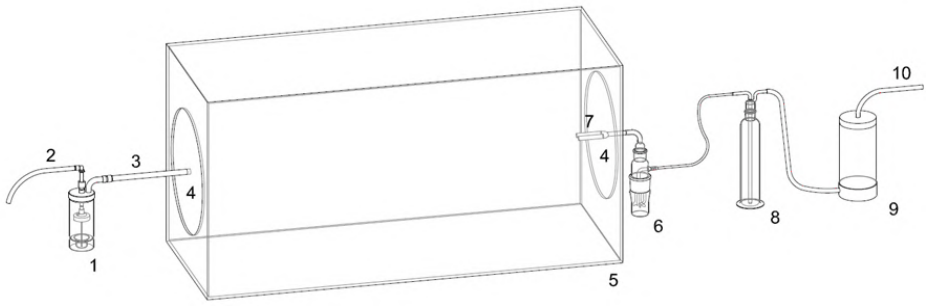

Figure 8. 1. CH-Technology BLAM aerosol generator; 2. Connection to compressor (TCR-TECORA Aero Particle Generator) 3. tube connection to 5. Bioaerosol Chamber: 4. side circular bioaerosol chamber closures; 6. SKC BioSampler; 7 Y shape bioaerosol inlet (internal to the bioaerosol box; 8. NaClO trap; 9. Air essicator/drier ; 10. connection to pump (TCR-TECORA BioBravo).

The test chamber is positioned within the Class II biosafety hood in a BSL3 laboratory, together with BLAM generator and SKC Biosampler with backup NaClO impinger and drier for protecting the sampling pump (as described in detail in [23]). This setting represents the second higher level of viral containment, positioned in a BSL3 laboratory (third higher level of containment).

The SKC Biosampler collects the bioaerosol from the chamber through a 3D-printed Y shaped PTFE tube sampling port simulating nostrils (small internal diameter 10 mm; large size i.d. 15 mm; length 80 mm) that can host the endonasal filters to be tested. NOTE: in advanced experiments, it can be connected to the SKC Biosampler by different length tubes (inert glass or PTFE) modifying distance between bioaerosol generation port and Y sampling port, allowing simulation of different distances between the infected emitter and the susceptible receiver in a poorly dispersive environment. Distances can be set, e.g., as 25 cm, 50 cm, 95 cm.

Proper directional airflow through the safety cabinet containing the test chamber, and the sealability of the test cabinet can be checked by using smoke tests. The relative humidity (RH) and temperature in the test chamber within the safety hood are at 75 ± 15% and 20 ± 1 °C, respectively. The BLAM nebulizer is charged with 6 mL of virus suspension to generate droplets/aerosols, emitted continuously for 15 min, simulating transmission from an infected individual. This time point is chosen, as the ECDC, CDC and WHO guidelines indicated that 15 min are the cut-off to define the

close contact between COVID-19 positive individuals and susceptible persons in a closed environment, without PPE [25,26,27]. Virus suspensions can be (1) 105 PFU/mL (~107 RNA viral copies/mL) to simulating very high viral load [23,28,29] and/or (2) 104 PFU/mL (~106 RNA viral copies/mL), considered by [26] in simulations for risk assessment. The number of particles emitted by the 8-Jet BLAM nebulizer operated as Multi Pass Atomizer is approximately one million and is compatible with the number of particles generated by multiple coughs or by a single sneezing. The size distribution from the aerosol generator is described in [21] with modes at 0.5 and 2 μm. The SKC Biosampler sampling flow (12.5 L per minute) is comparable to inhalation during moderate activity, and it occurs through the 3D-printed Y shaped PTFE tube sampling port (Figure 9). The section of PTFE tube can be 15 mm or 10 mm, simulating wider or narrower nostrils that host different sizes of endonasal filters (Figure 3).

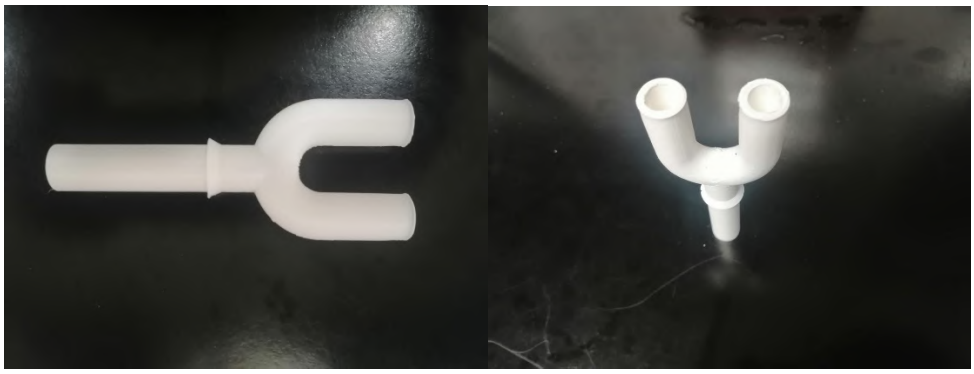

Figure 9. Photographic images of the Y shaped tube sampling port

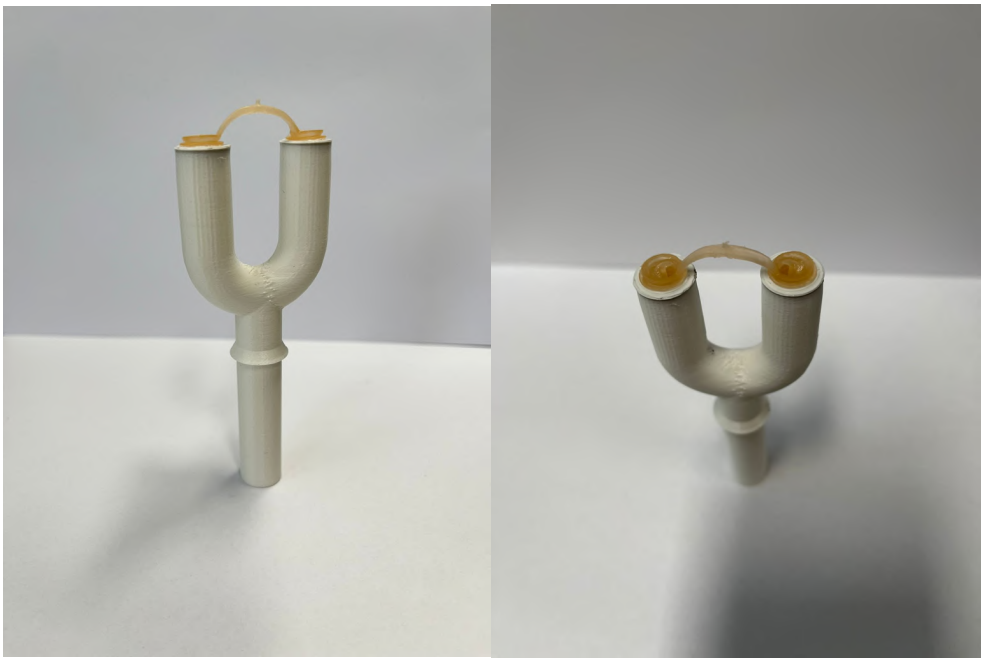

Figure 10. Photographic images of the Y shaped tube sampling port with the endonasal filters

A first set of experiments (without the filters) is necessary to test the system's efficiency in virus aerosolization and collection and would simulate a situation of close contact without PPE. The more diluted virus suspension is tested first to reduce the probability of carry-over. Three replicate experiments are performed for each of the two virus suspensions. BLAM nebulizes virus for 15 min and contemporaneously the SKC bio-sampler collects the aerosol from the chamber through the 3D-printed Y shaped PTFE tube sampling port. One of the removable side circular bioaerosol chamber closures hosts a hole with a 0.2 um filter to allow balance in the aerosol chamber between inlet (aerosol generator, 8 lpm) and outlet (sampler, 12.5 lpm) flows.

Between the experimental replicates, the BLAM and the SKC bio-sampler reservoirs are carefully washed with sterile water; moreover, the chamber and the collecting tubes are flushed for 5′ with 75% ethanol and 5′ with sterile water to avoid carryover between the replicates. A further 10' of air flushing is added in order to remove ethanol residuals from the bioaerosol chamber. At the end of the replica tests, the chamber and the collecting tubes are flushed for 15′ with 75% ethanol and 15′ with sterile water and the chamber is cleaned internally with ethanol and water. Prior to each experimental replica, the presence of viral RNA in the SKC Biosampler will be tested in order to verify the correct disinfection of the apparatus.

A second set of experiments is needed to test the effectiveness of the endonasal filter in preventing the droplet/aerosol transmission of virus. A couple of endonasal filters (Small/Large size) are inserted into the two branches of the Y shape connector of 10–15 mm internal diameter to prevent the droplet/aerosol transmission of viable virus to the Biosampler collection liquid. BLAM nebulizes virus for 15 min and contemporaneously the SKC bio-sampler collects the aerosol from the chamber through the 3D-printed Y shaped PTFE tube sampling port. Three replicate experiments are performed with endonasal filters on the sampling line for each of the two virus suspensions. To prevent cross-contamination of the infectious virus and viral RNA, the chamber and the collecting tubes are flushed for 5′ with 75% ethanol and 5′ with sterile water and all filters are disposed after each experimental trial. Virus aerosolization and quantification follow [21].

2.2.1 SARS-CoV-2 suspension preparation

The SARS-CoV-2 selected variant isolated, amplified and quantified on Vero E6 cell line, is employed in the aerosolization [30,31,32]. The virus is diluted at 105 and 104 PFU/mL for aerosol generation in the infection medium composed by MEM supplemented with 2 mM glutamine, 2% fetal bovine serum and 100 U/mL penicillin/streptomycin.

2.2.2 The bioaerosol measuring train

Experiments on residual infectivity after virus aerosolization are conducted in a class II biosafety cabinet within a BLS3 facility, in a sealed experimental bio-aerosol chamber assembled as described above. A BLAM aerosol generator hosting the SARS-CoV-2 suspension in its precious jar received an air flow produced by an AERO Particle Nebulizer pump (TCR Tecora Srl—Cogliate, MB, Italy) positioned outside the cabinet which generate the bio-aerosol. The BLAM has a filtered inlet of 0.2 μm, allowing entrance of air needed to sustain the air flow in the measuring train. The aerosol is transferred into the bioaerosol chamber described above, and then to a Biosampler (SKC Inc., Eighty Four, PA, USA), which collect the aerosolized SARS-CoV-2 into a dedicated vessel. An aspiration flow of 12.5 L per minute generated by a Bio-Bravo sampling pump (TCR Tecora Srl, Cogliate, MB, Italy) sustains the virus particle collection into the Biosampler. A glass impinger, with sodium hypochlorite working as safety trap deactivating eventual unsampled pathogens, and a silica dryer are positioned online between the Biosampler and the Bio-Bravo to protect the pump from vapors.
All the connections are prepared in nylon with o-rings. The bio-aerosol measuring train (Figure 1) has been set up and tested with *E. coli* BL21-DE3.

2.2.3 Aerosol generation

The selected bioaerosol generator is a Blaustein Atomizing Modules (BLAM) nebulizer (CH Technologies Inc., Westwood, NJ, USA) with 8 jets, in consideration of superior nebulization efficiency [33] in comparison to common Collision nebulizers or the Sparging Liquid Aerosol Generator (SLAG). The BLAM generates less significant impacts of the bioaerosol on hard surfaces and requires lower pressures than the Collision nebulizer, provoking less stress to the microorganisms, with a resulting inferior loss of infectivity. The virus suspension (~5 mL) is added in the precious jar of an 8-jet BLAM nebulizer with horizontal discharge, operated in Multi Pass Atomization mode, at a flow of 8 L per minute (lpm). The aerosol is released in a 1000 × 500 × 400 mm parallel-piped bioaerosol chamber, subject to aspiration from the Biosampler (12.5 lpm); the duration of aerosolization is 15 min. Filtered air required for equilibrating flows enters into the system through a 0.2-micron Poly-ethersulfone filter fitted onto the BLAM cap and on a further hole on the removable side closure of the parallel pipes. After aerosolization, the remaining liquid is collected in a falcon tube and the chamber is washed with water; after that, the viral suspension for the next run is added.

2.2.4 Size distribution temperature and relative humidity assessment

The size distribution of aerosol particles generated from the infection medium (MEM + 2% fetal bovine serum; 2 mM glutamine; 100 U/mL penicillin/streptomycin) by the BLAM nebulizer is measured in dedicated runs at the inlet and outlet of the parallel-piped aerosol chamber, with 8 lpm aspiration from TCR Tecora Bio Bravo, to check size distribution and eventual modifications. An Optical Particle Counter measures size distribution (e.g., GRIMM EDM) counting 107 particles in the range 0.25–32 μm, with acquisition time of 6 s. Relative humidity (RH) and temperature are measured using a thermo-hygrometer (e.g., Bluetooth connected Inkbird IBS- TH1 Plus), located in the chamber.

2.2.5 Bioaerosol sampling

For the collection of viable viruses from aerosol, a swirling aerosol collector—e.g., BioSampler (SKC Inc., Eighty Four, PA, USA)—connected by the Y shaped tubing to the end of the parallel-piped aerosol transmission chamber is used for sampling bioaerosol in 20 mL of infection medium. The sampling duration is 5 or 10 min. The sampling flow is 12.5 lpm sustained by a TCR Tecora Bio Bravo pump. The pump is protected from microbial contamination by an impinger containing NaClO as a disinfectant and by a silica dryer that treats the sucked air. At the end of the procedure, the liquid sample is collected in a falcon tube; the sample container is washed in distilled water and refilled with a fresh medium.

2.2.6 Infectivity Assessment

The infectivity of the bio-aerosol samples is determined in vitro on Vero E cells (epithelial kidney standard cell line derived from Cercopithecus aethiops). Vero E6 cells still represent a gold standard for viral infection experiments and are highly susceptible to SARS-CoV-2 infection [31].

Vero E6 cells are maintained in MEM supplemented with 2 mM glutamine, 10% fetal bovine serum and 100 U/mL penicillin/streptomycin and seeded on 6 multi-well plates (350.000 cells for well). During the infection phase, the cells will be cultured in 2% FBS. Briefly, after the sampling, the liquid collected at the end of the experimental setting is filtered with a 0.2-μm filter in order to eliminate bacteria and impurities potentially present in the apparatus.

The samples are seeded on a monolayer of Vero E cells and monitored by the optical microscope for 6 days to assess morphological modifications, e.g., cytopathic effects (CPE) characterized by cell vacuolization, rounding and detachment.

The viral load in the supernatant is determined through semi-quantitative real time PCR (qRT-PCR) daily. The RNA is extracted through thermolysis; 15 µL of the supernatants is mixed with 45 µL of distilled water and heated at 98 °C for 3′, followed by 5′ at 4 °C. The samples are then stored at −80 °C to avoid RNA degradation until analysis.

The intracellular RNA is extracted with an RNA Zymo RNA extraction kit (Zymo Research, Irvine, CA, USA). qRT-PCR is performed with The Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, USA) with the primers and probes targeting gene N, E and sub-genomic E (the last to detect the viral replication intermediates indicative of active viral amplification inside the cells [34,35]) as reported in Table 1.

Table 2. Primers and probe for quantification of RNA viral load (qRT-PCR).

Name	Sequence (5' -> 3')	concentration	Label
2019-nCoV_N1 Forward primer	GAC CCC AAA ATC AGC GAA AT	500 nM	
2019-nCoV_N1 Reverse primer	TCT GGT TAC TGC CAG TTG AAT CTG	500 nM	
2019-nCoV_N1 Probe	ACC CCG CAT TAC GTT TGG TGG ACC	125 nM	FAM BHQ-1
2019-nCoV_N2- Forward primer	TTA CAA ACA TTG GCC GCA AA	500 nM	
2019-nCoV_N2 Reverse primer	GCG CGA CAT TCC GAA GAA	500 nM	
2019-nCoV_N2 Probe	ACA ATT TGC CCC CAG CGC TTC AG	125 nM	FAM BHQ-1
E gene Forward primer	ACAGGTACGTTAATAGTTAATAGC GT	400 nM	
E gene Reverse primer	ATATTGCAGCAGTACGCACACA	400 nM	
E gene Probe	ACACTAGCCATCCTTACTGCG	200 nM	FAM BHQ-1
subgenomic E gene Forward primer	CGATCTCTTGTAGATCTGTTCTC	400 nM	

A plaque forming unit assay is also performed.

100 µL of the collected sample is seeded in triplicate on a monolayer of Vero E6 cells, then after 1 h the cells are overlaid by immobilizing medium composed by 1:1 DMEM + 4% FBS: carboxy methyl

cellulose (CMC) 4% and incubated until the plaques are visible (4–6 days). At the end of the 4–6 days, 1 mL of PBS is added in each well to remove the immobilizing medium. After washing, the cells are fixed with paraformaldehyde 4% in PBS for 20 min and stained with 0.1% crystal violet in PBS (Merck KGaA, Darmstadt, Germany) for 1 h. After 3 washes in water, the plates are air-dried, and the plaques formed are counted.

2.2.7 Viral RNA Load after Aerosolization

SARS-CoV-2 RNA viral load is measured in the liquid present in the initial suspension, in the nebulizer after aerosolization and in the aerosol samples collected in the SKC bio-sampler, by the procedure described in the previous chapter.

2.3 Statistical analysis

Each experimental setting (viral load; size of endonasal filter) is replicated three times and for each replica set, median (average) and standard deviation are calculated.

The efficiency of aerosolization is determined by evaluating the difference between the RNA viral load in the nebulizer (initial viral concentration) and in the SKC bio-sampler at day 0 (day of experiment).

The efficacy of virus filtration is calculated as the RNA viral load ratio between the collection of viruses with and without filters applied to the port of the collection tube of the SKC bio-sampler at day 0 (day of experiment). Other time points are also tested.

The growth curves, measured daily through molecular tests, are compared between the virus collected with and without filters through linear regression analysis.

The intracellular viral load at day 7 (end of the experimental planning) and the results from PFU assay between the virus collected with and without filters is compared by Mann-Whitney test.

3 Evaluation Outcomes

The principal aim of the study is the setup of safe apparatus and procedure for the assessment of the filtration power of bio-gel silver nanoparticle (AgNP) endonasal filters on SARS-CoV-2 viruses.

Evaluations at different stages of the procedure are mandatory to define the performance of our system and represent the checking points for the quality assessment of the study protocol.

The efficiency of the aerosolization and collection systems will be assessed on the first day of experiment, as well as the efficacy of virus filtration by endonasal filters. The assessment is determined by the measurement of RNA viral load between the reservoir of the nebulizer and the jar of the air-sample and between the samples collected by air-sampler with and without the endonasal filter.

The viral load abatement resulting from endonasal filter application would be shown as the percentage of the RNA level reduction.

Other time points are studied in order to determine if the endonasal filters block infectious virions. Indeed, the molecular determination of viral RNA does not underline the infectious potential of the sample collected.

To achieve this task the samples collected in the SKC bio-sampler are seeded on Vero E6 cells and monitored daily. The presence of cytopathic effect, the formation of plaque in the PFU assay and the increment of the viral load in the supernatant are all indicators of virus amplification that will be taken into consideration in the study. The presences of viral genomic RNA and of sub-genomic RNA intracellularly are also assayed. The sub-genomic RNA is an intermediate of intracellularly viral replication machinery and a useful marker to check the virus replication.

4 Discussion

The protocol here presented would suggest some basic useful information to set up a method for testing PPE in a dynamic status (i.e., the nebulization of the virus and aerosol sampling) mimicking as much as possible a real-life situation.

SARS-CoV-2 spreading has been exhausting health system capacity. Although the predominant Omicron variants have been leading to more attenuated symptoms and to the reduction of the hospitalization rate, the ability of Omicron SARS-CoV-2 to evade the immune system greatly impacts on the continuously increased number of infected individuals [1,2,3].

Therefore, the need for testing PPE to counteract virus spread continues to be a challenging topic. Moreover, there is no standard procedure for the quality control of PPE, employing infectious virus and dynamic conditions.

The main result that this study will achieve is the setting up of a standardized method to test PPE in laboratory-controlled conditions.

Our study protocol will accurately describe all the steps of the procedure to analyze PPE (in this case bio-gel silver nanoparticle (AgNP) endonasal filters) efficacy in blocking the virus passage.

The procedure here characterized will strictly simulate the aerosol emission from an infected person (BLAM nebulizer with a flow of 8 L per minute) in a close environment (the aerosol chamber) for the period of close contact, definition (15 min), to a susceptible host (the PTFE tube sampling port, 10 or 15 mm simulating wider or narrower nostrils, connected to the SKC bio-sampler with a flow of 12.5 L per minute).

By employing this setting, the efficiency of the system in generating and collecting bio-aerosol can be defined as well as the performance of virion retainment by the bio-gel silver nanoparticle (AgNP) endonasal filters.

We expect that this study protocol can be easily translated into further PPE testing applications and will contribute to defining standard conditions for dynamic quality control. Indeed, the experimental setting may be modified for assessing filters or masks optimized for children (pediatric applications) and teenagers, by modulating the aspiration of the samplers (less than 12.5 lpm) and using filter holders with a lower section; such variations can modify inner flows in the filters and the effectiveness of interaction of bioaerosol/airborne pathogens with the AgNP coated walls. It will also be possible to simulate the inhalation of children, adolescents, adults or the elderly, with screening conditions typical of different ages.

The main limit of the study is related to the controlled laboratory condition tested. Our setting is a simulation of a real-life situation and the absolute efficacy of endonasal filters can only be tested live. Nevertheless, our system can be very useful in obtaining indications on the efficacy of endonasal filters in blocking virus passage, at least in vitro. Moreover, all the steps (virus concentration, virus aerosolization, aerosol sampling, duration of the test, flow speed) are carefully selected in order to be as similar as possible to a critical real indoor environment. Another limit is related to the traslatability of the results in a real setting, in terms of virion numbers that are really able to infect the host and so produce illness.

Up to now, no standard test method is available for assessing viral filtration or inactivation efficiency of PPE for airborne SARS-CoV-2 [13]. An adapted protocol testing viral filtration and inactivation efficiency of face masks has been proposed by Nelson Laboratories starting from a Bacterial Filtration Efficiency test ASTM F2101-14 and using PhiX174 virus as a challenge [36], with mean particle size of 3.0 ± 0.3 μm and collection of the bioaerosol on a six-stage Andersen impactor; PhiX174 is also

an enveloped virus considered a possible nonpathogenic surrogate for SARS-CoV-2 [37], and MS2 bacteriophages have been proposed [15,38], or inactivated virus [17].

Viral residual infectivity transmitted through masks or filters can be more properly assessed by aerosolization of viruses of interest, without resorting to surrogates. Like our protocol, a work by Ueki et al. [24] illustrated an airborne transmission simulator, where the air was sampled by using gelatin membrane filters and different types of masks were tested. Interestingly, SARS-CoV-2 was able to pass through cotton and surgical masks but also through N95 masks and fitted N95 (completely fitted with adhesive tape to the mannequin head), although the researchers showed a barrier effect for all the types of PPE, higher when the mask was worn by the spreader. As the reduction is expressed in terms of logarithmic viral load (tested both as PFU/mL and RNA viral copies/mL), it can be easily observed that the percentage of filtration performance should be high, especially when infectious virion numbers are considered. Indeed, viral RNA can be derived from non-infective viruses or damaged virions. The results reported are quite interesting considering that the presence of the N95 mask only on the receiver slightly reduced the collection of the virus on the gelatin filters placed after the mask and that the greater reduction was determined when the mask was fitted with “adhesive tape” (a condition that does not sum up well a real situation). Nevertheless, the very close space in which the mannequins are placed is a limitation of the study by Ueki et al. (0.24 m3) and of our study protocol that should be considered in the evaluation of circulating viruses. In comparison with the Ueki simulator, the proposed aerosol chamber coupled with the BLAM generator can allow the modulation of the viral challenge for endonasal filters; moreover, the SARS-CoV-2 collection in the liquid by swirling sampler improves the capability of correct infectivity assessment, with and without personal protective filter, due to reduced mechanical impact between bioaerosol and collection medium [39].

5 Conclusions

A first proposal for a procedure and apparatus is presented for assessing SARS-CoV-2 Viral Filtration Efficiency capable of testing endonasal filter performances.

The apparatus could be further implemented for more sophisticated studies on contaminated aerosols, mimicking in more realistic way human respiration and acting as sneeze or cough (e.g., by CHεST aerosol emission controller), or studying the evolution in time of bioaerosol within the chamber/apparatus.

Having a testing procedure can sustain application and spread eventually to further research and optimization of this highly promising personal protective equipment that presents potential applications in both adult and younger populations.

6 Further developments

A private company asked for application of the testing setting; results have been produced by BAQlab but are protected by a non-disclosure agreement. An improvement to the published procedure could be achieved implementing a pump simulating the sinusoidal rhythm of respiration that could generate turbulence within nasal filters, similar to physiological conditions, favoring collision of bioaerosol to filter inner surface. In a recent study Saravanan and colleagues used the QuickLung Breather device to impose a rhythm mimicking human respiration during bioaerosol sampling [40].

7 References

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SECTION 2

Characterization and optimization of a novel UVC-LED aerodynamic device for airborne microbe viability abatement

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Published on *ACS ES&T Engineering*, https://doi.org/10.1021/acsestengg.4c00052

Abstract

The escalation of the COVID-19 outbreak has significantly increased research into the transmission of airborne infectious diseases in indoor settings, underscoring the urgent necessity for affordable and efficient methods of air disinfection. The aim of the present work is the development of a complete framework based on designed experiments for exploring and optimizing the bioaerosol removal and inactivation efficiency of a novel air disinfection device. This device combines the aerodynamic effect of a three-dimensional vortex structure with UV-C radiation provided by commercially available UV-C light-emitting diodes (UV-C LEDs). The system was designed and tested to locally maintain a high radiation intensity that is suitable for bioaerosol disinfection. A controlled experimental laboratory model of bioaerosol aerosolization was set up by using an impinger medical vibrating nebulizer, a cylindrical chamber for bioaerosol travel, and an SKC BioSampler for collecting microorganisms capable of replicating. A nonpathogenic strain of *Escherichia coli* (BL21-DE3) was used as a model of airborne bacteria. The inactivation efficiency was assessed based on the enumeration of the colonies originating from viable *E. coli*. Interactions between analytical factors and their optimal levels were investigated by using sequential D-optimal designs adapted to domain constraints and previous computational simulations of the aerodynamic performance of the device. Five experimental variables (the concentration of aerosolized bacteria; the size of aerosol particles; the volumetric airflow; the power of the LEDs; and two configurations of the device) were considered as factors in the optimization process. Response surfaces allowed for the identification of the ideal working conditions to maximize the efficiency of the device, an essential requirement for the device's future exploitation in real-world settings.

1 Introduction

The spread of COVID-19 has intensified studies related to airborne infectious disease transmission in indoor environments, highlighting the critical need for cost-efficient and effective air disinfection solutions [1]. The indoor air microbiome is composed of bacteria, fungi, viruses, and metabolites originating from these agents. These microorganisms are either suspended in the air or attached to inorganic particles such as dust, ash, mist, and particulate matter (PM), forming what is known as a bioaerosol. Typically, the microbial load in the bioaerosol and its residence time may decay exponentially with travel time or droplet size. However, substantial levels of bioaerosols can still arise and gather in public spaces or enclosed settings with limited ventilation, such as airports, schools, offices, train stations, and hospitals [2]. The raised awareness regarding the airborne spread of various respiratory illnesses including tuberculosis, SARS, measles, and influenza, [3] coupled with the newfound understanding of air as a key vector in the dissemination of antibiotic-resistance genes and antibiotic-resistant bacteria, [4] has spurred a growing demand for innovative and cost-effective technologies and materials [5−7] capable of reducing the concentration of pollutants [8−10] and viability of airborne pathogens [11]. Considerable efforts have been dedicated to the exploration of engineering methodologies aimed at effectively mitigating the presence of airborne pathogens inside building environments with limited air circulation. Several strategies for sanitizing indoor air have been studied, but none of them have resulted in the establishment of universally accepted air treatment protocols [12].

Available solutions for air treatment rely on a variety of technologies that can be roughly categorized as either filtration technologies or inactivation technologies, either separately or in combination [13−16]. A technique often employed in contemporary practice is the filtration of air within heating, ventilation, and air conditioning (HVAC) systems, supported by established classification and criteria (e.g., ANSI/ASHRAE Standard 52.2–1999 and ISO 14 644–1 1999) [17,18]. High-efficiency particulate air (HEPA) filters are based primarily on mechanical filtration, i.e., reducing the size of the pores through which the air flows to the size of the particles to be trapped. They can remove 99.97% of particles with a diameter of 0.3 μm or greater. Although this methodology is reliable and well established, it has two main drawbacks: the pressure loss introduced in the air circuit needs to be overcome by forced ventilation with consequent energy consumption; the porous media must be maintained and frequently be replaced or regenerated while need to be handled as a contaminated item [19]. Poorly maintained HEPA filters in HVAC systems could result in the accumulation of viable pathogens and secondary transmission through air

circulation [2]. However, elimination strategies encompass the utilization of UV radiation, ozonation, photocatalytic oxidation, electrostatic precipitator, and other techniques, which can also be employed in conjunction [13−16]. The UV-C LED light with wavelengths ranging from 200 to 280 nm is regarded as one of the extensively researched techniques for sanitizing indoor environments. The efficacy of UV-C lamps put in HVAC systems for ultraviolet germicidal irradiation (UVGI) has been established in effectively neutralizing airborne infections [20]. In addition, the use of upper room UV-C lamps is being proposed for disinfection also in living spaces, despite rather limited safety evidence [21]. The efficacy of UV-C disinfection is contingent upon various factors, including the intensity of the UV source, the duration of exposure, the proximity of microorganisms to the source, the resistance of microorganisms, the presence of particulate matter, and the existence of shadow zones, among others [22]. Thus, the efficient operation of UV-C devices in HVAC systems requires a comprehensive understanding of the complex interplay between fluid dynamics, radiation distribution, and microbial susceptibility to irradiate UV-C against pathogens for the time necessary for obtaining uniform and efficient microbial abatement [23,24].

Currently, there are no air treatment strategies available for inactivating airborne viruses during hospital outbreaks, which is due to the lack of approved protocols. The need for quantitative studies on factors affecting air sanitation is evident, as it will serve as a crucial next step in benchmarking technologies and approaches [25].

In this paper, we propose an integrated approach introducing and optimizing a novel device designed to combine the aerodynamic effect of a three-dimensional vortex structure with UV-C radiation. Although these two combined technologies have been applied in air purifier equipment before this study, the characteristics of the device's structure and material and UV-C inactivation of bioaerosols were not fully investigated. With the right interplay between the internal geometry of the device and the properties of the materials used, it is possible to confine the microorganisms in bioaerosols within the device for a long enough time to render them inactive through exposure to UV-C radiation without experiencing any pressure loss. In this study, a nonpathogenic strain of *E. coli* (BL21-DE3) was used as a model of airborne bacteria to investigate the effects of varying parameters of the combined technologies for the inactivation of bioaerosols in a controlled experimental setup. A systematic and data-driven methodology merging the strengths of computational fluid dynamics (CFD), ray tracing simulations, and chemometrics was, therefore, used for the first time to bridge the gap between theoretical insights and practical implementation. In this way, almost all parameters that influence the performance of the system were investigated and optimized.

2 Materials and methods

2.1 Materials

All solvents were of analytical grade and used without further purification. Milli-Q water was prepared with a Millipore apparatus. All reagents were handled according to their respective safety data sheet. The BL21(DE3) chemically competent cells, lysogeny broth (LB), LB broth with agar, and phosphate buffer saline (PBS) tabs were purchased from Sigma-Aldrich (Merck). The sealing parts of the train with O-rings were prepared in poly(tetrafluoroethylene) (PTFE) by the mechanical workshop of the Department of Chemical and Pharmaceutical Sciences of the University of Trieste, while filters and connectors were designed and 3D-printed in polylactic acid (PLA) by ESTECO.

2.2 Device design and simulation

The device geometry shown in Figure 1 was described in Ref. [26,27] and was originally patented (Italian patent n.0001401336) as a grease-removing device for industrial kitchens. As reported in Ref. [26,27], the original optimized geometry was capable of separating grease particles having 4–6 μm diameter. Here, the geometry was scaled to interact with smaller particles and to fit with the available experimental apparatus. The internal walls were made of highly reflective POREX Virtek PTFE, which offers a certified reflectivity of 97–95% in the 200–300 nm wavelength. At the opposite side of the device module, 2 UV-C LEDs were positioned and controlled with a dedicated adjustable power supply, allowing the independent modulation of the two. The aerodynamic performance of the device has been verified using a Navier–Stokes solver simulating the nominal flow rate of 12.5 L/min of the experimental apparatus using a k-w turbulence model and steady-state flow. The particle path has been modeled using Lagrangian transport of spherical droplets having diameters of 0.5, 1.5, and 2.5 μm, as those produced in the experiments (Table 1).

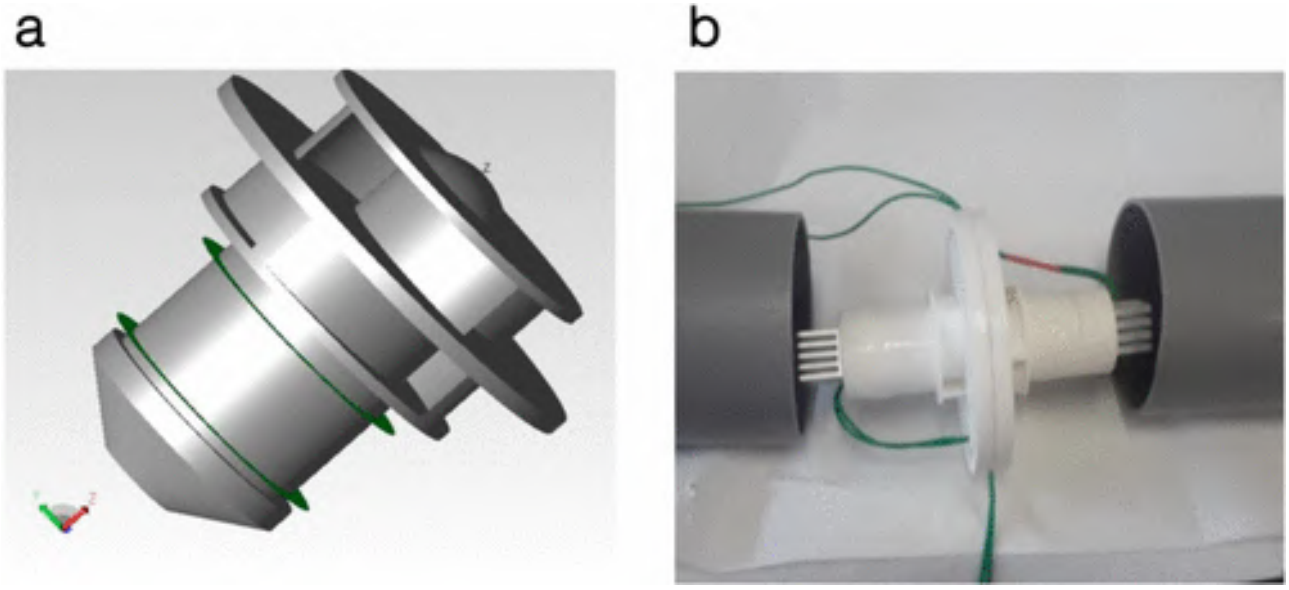

Figure 11. Device geometry. (a) 3D view of half of the device. (b) Assembled prototype used for the experimental testing.

Table 3. Residence time of particles of different diameters inside the device.

Particles diameter (μm)	Residence time (s)
0.5	0.40 ± 0.07
1.5	0.37 ± 0.20
2.5	0.41 ± 0.15
5	0.38 ± 0.09

Wall–particle interaction has been modeled both using a perfect rebound and with a wall film formation model to estimate the residence time of the aerosol droplets in the device. The LED emission pattern and the emitted power have been modeled according to the related datasheet at the current of 200 mA, while the wall reflectivity has been set to 97% as suggested by the POREX Virtek datasheet.

2.3 Preparation of microbial liquid cultures

A nonpathogenic strain of *Escherichia coli* (BL21-DE3) was used in this study as a representative model of sensitive bacteria present in the air in many indoor and occupational environments. (28,29) The preparation of liquid cultures was completed on the day before the experiments. The strain was grown overnight in LB, containing 10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, and 5 g/L NaCl (Sigma-Aldrich) maintained at 37 °C and 180 rpm shaking. The overnight culture was refreshed in

LB and grown until the desired optical density (OD600, 0.30 ± 0.03) was reached. The bacteria were then diluted in PBS (0.015 M, pH 7.4) to reach the final concentrations necessary for the experiments (of approximately 1 × 107 and 1 × 108 CFU/mL). These suspensions were subsequently used to produce experimental bioaerosols.

2.4 Experimental setup

The experimental setup was based on the simple setup for laboratory studies that we designed for the understanding of the behavior of infectious SARS-CoV-2 in aerosol phase and described in our previous work [30]. We modified this previous bioaerosol measuring train with the insertion, in the confined aerosol path, of the aerodynamic device subject of the study. The experimental setup is illustrated in Figure 2. Briefly, a compressed air generator (TCR Tecora Srl, Cogliate, MB, Italy, mod. DDS Aero Particle Nebulizer) at its maximum capacity was connected to a medical aerosolization device. The air flowed into the medical aerosolizer through a nozzle gurgling inside it, impacting the liquid and increasing the surface tension, thus creating the aerosol. This instrument allowed the generation of particles of different dimensions (0.5 and 2.5 μm) from the PBS solution enriched with a culture of *E. coli*, at two different concentrations: 107 and 108 CFU/mL. The particle dimensions generated by the aerosol system were evaluated by a light scattering-based particle counter (GRIMM EDM 107, Grimm Aerosoltechnik, DE).

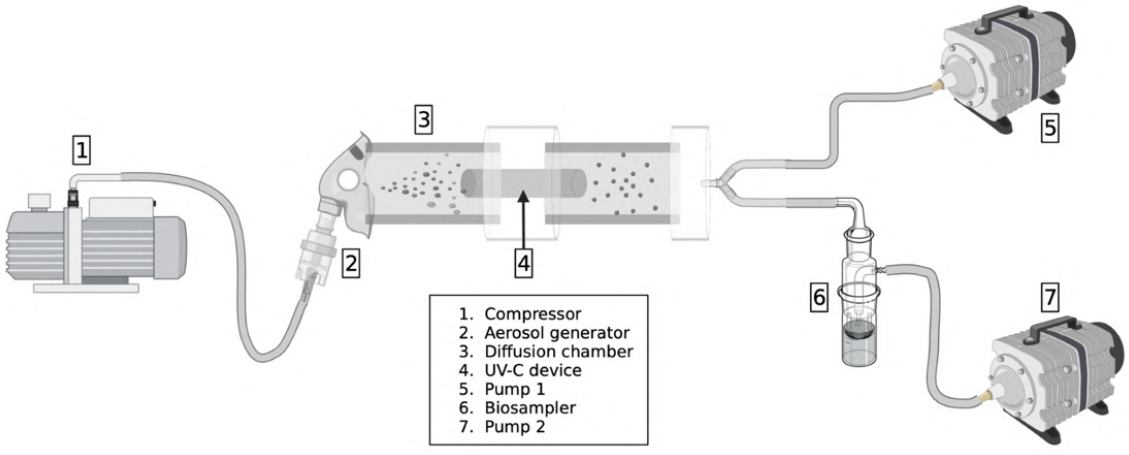

Figure 12. Schematic diagram showing the experimental setup. A medical aerosolization device (2) receives airflow from the Aero nebulizer pump (1) and generates the bioaerosol that is transmitted through the aerosol diffusion chamber (3) hosting the UV-C device (4), and it is collected in the vessel of the BioSampler (6) before entering a BioBravo pump (7) that sustains the sampling. The main airflow is regulated by a BioBravo pump (5).

The generated particles were then transferred into the diffusion chamber, a hollow cylinder in poly(methyl methacrylate) (PMMA) of 300 mm length and 75 mm diameter, containing the new aerodynamic device in the middle section.

The diffusion chamber had at its end a U-shaped tube, which made it possible to connect at one extremity a SiO2 desiccator connected to a high-volume suction pump (model BioBravo, TCR Tecora). This allowed to increase the flow rate of the system up to a total of 50 L/m. From the other extremity, the airborne particles were downstream sampled by the BioSampler (SKC Inc., Eighty Four, PA, USA), a liquid-based impinger widely used for bioaerosol sampling [31]. It consists of an inlet, through which airborne particles pass into the collection device, and an outlet, connected to a second high-volume suction pump. The flow rate of the BioSampler was maintained at 12.5 L/min during all the tests, as suggested by the manufacturer. In the collection vessel, three tangential nozzles ensured a swirling motion of 20 mL of the collection liquid upward on the inner walls. This peculiar design minimizes reaerosolization and bounce of particles, preserving the integrity and viability of microorganisms like viruses, bacteria, fungi, and molds [32,33]. The collection liquid used was a PBS solution, which could be easily transferred to agar plates for culturing. All samples were collected at different experimental conditions (see the following paragraph for details) every three minutes, adding new collection liquid into the collection vessel. After each run, 25 µL of solution collected in the BioSampler was seeded on a 6 cm LB agar Petri dish and incubated overnight at 37 °C. Fresh collection buffer was used for each sampling condition. All experiments were conducted at room temperature (24 °C ± 1.2), and after each run, the sampler was carefully washed with pure distilled water; moreover, the experimentations started from the less concentrated bacterial suspension to avoid any potential unintentional contamination and residual carryover. The day after the incubation, individual colonies from countable plates were counted and used for inactivation rate calculations. The inactivation efficiency rate (ε) was calculated as follows:

$$\varepsilon = 1 - \frac{C_{sample}}{C_{control}} \times 100$$

where C_{sample} and C_{control} represent the colony number (CFU/mL) for each experimental run (sample) and for the control (LED off), respectively.

2.5 Design of Experiments

To assess the performance of the device across various conditions, a stepwise sequential optimal experimental design was devised, permitting newly acquired data to guide the design of future

experiments. Initially, five quantitative process factors were investigated as possibly affecting the inactivation rate of the device: the bacterial concentration, particle dimensions, air flow, and power of the two LEDs, independently operated. The levels to be tested were chosen after a preliminary screening of the relevant information available in the technical literature, according to the results from the device simulations and the instrumental operational limits. A complete description of the factors and levels is reported in Table 2.

Table 4. Experimental Factors and Levels for the First Experimental Design

Coded variables (X_i)	Factors	Unit	Levels
X_1	*E. coli* concentration	CFU/mL	10^7, 10^8
X_2	LED 1 power	mA	0, 100, 200
X_3	LED 2 power	mA	0, 100, 200
X_4	Aerosol particle size	µm	0.5, 2.5
X_5	Air flow	L/min	12.5, 25, 50

The response variable, inactivation efficiency rate (ε), was taken to be a function of the five factors, considering quadratic terms for the power of the two LEDs and the airflow and all two-factor interactions:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 + b_{22}X_2^2 + b_{33}X_3^2 + b_{55}X_5^2$$

where Y = response; b0 = constant; b1, b2, b3, b4= linear term coefficients; b12, b13, b14, b23, b24, b34, = first-order interaction term coefficients; b33, b44 = quadratic term coefficients. Also in this case, the response surface methodology (RSM) was used to study the influence of the input factors on the response within the design space [34,35].

2.6 Chemometric tools and software

Numerical modeling of the particle flow in the device was conducted using commercial computational fluid dynamics (CFD) simulation software ANSYS Fluent (2021R1). UV-C LED exposure was modeled using TracePro (Version 2020_20.1 Lambda Research, Littleton, CO, USA). Design of experiments and preliminary data analysis were performed using modeFRONTIER (version 2021R1 ESTECO SpA, Trieste, Italy). Further data processing and visualization were performed within the R software environment for statistical computing and graphics (R version 4.1.2

"Bird Hippie"), building on the packages *rsm* [36] on a commercially available workstation (x86_64-apple-darwin17.0 64-bit). In-house developed R scripts were used for visualization and further processing.

3 Results and discussion

3.1 Simulations

The residence time of aerosol particles inside the device directly influences the exposure time of these particles to UV-C light. Aerosol particles containing bacteria need to be exposed to a sufficient dose of UV-C light to ensure an effective inactivation. If the residence time is too short, then particles might not receive an adequate dose, resulting in incomplete inactivation. Conversely, if the residence time is too long, there is a risk of overexposing particles, which could lead to other unintended oxidation byproducts. CFD and raytracing simulations were used to evaluate a priori how changes in design parameters, such as geometry, UV-C emitter placement, and flow rate, affect the device's performance and if the UV-C radiation intensity inside the device is sufficient to achieve a proper disinfection. The trajectory of the particles through the device is shown in Figure 2. The particle trajectories were predicted by the Lagrangian method, and the particle flow trajectories are colored by the velocity magnitude (m/s).

Figure 3 shows the radiation intensity obtained in a preliminary simulation in three key sections of the device. Thanks to the adoption of a highly reflective optical material for the internal part of the device, a radiation intensity between 220 W/m2 and 600 W/m2 can be obtained inside the device. Considering the residence time in Table 1, this provides a preliminary approximation of the amount of UV-C radiation received by the particles, which is determined to be a minimum of 46 J/m2 (4.6 mJ/cm2). This level of UV-C dose is expected to ensure a microbial inactivation of at least 4 log, making it ideal for aerosols disinfection. (37) For in-duct UV-C disinfection, ASHRAE requests a minimum UV dose of 1.5 mJ/cm2 and a minimum UV exposure time of 0.25 s. (18) It should be noted that all simulations were conducted only during the design phase for the purpose of evaluating the feasibility of the project. It is evident that more precise calculations can be conducted at a later stage to accurately predict the performance of the device in a specific configuration. These calculations can be further utilized to optimize the performance of the device through simulation in subsequent studies or for a specific installation.

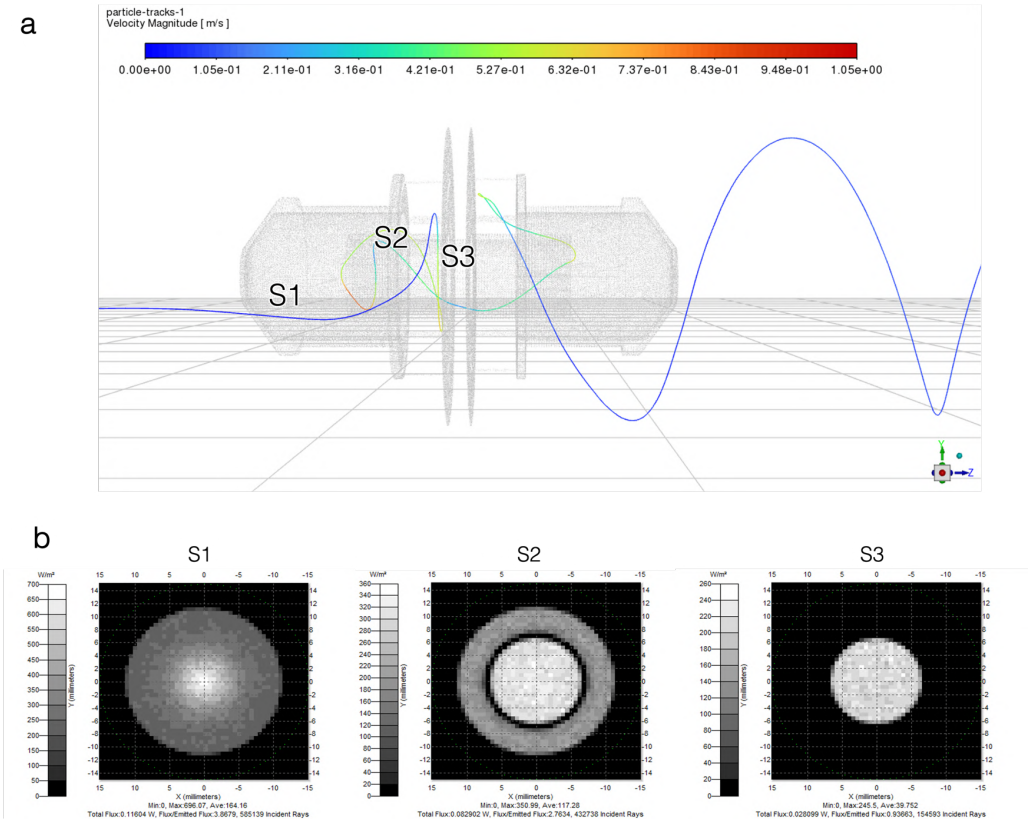

Figure 13. Computational simulations. (a) Particle flow path. (b) Spatial distribution of radiation intensity in 3 sections of the device.

3.2 Bioaerosol inactivation efficiency

Following the results of the CFD simulations, an optimization process was carried out. The goal of the experimental design was to determine the effects and possible interaction effects of a set of different process variables (factors) on the inactivation efficiency rate of the novel device. To facilitate experimentation, we evaluated the culturability of aerosolized *E. coli* as a straightforward approach to measure viability. *E. coli* is a nonspore-forming bacterium that can be easily dispersed in the air. It is convenient to handle (classified as biosafety level [BSL]–1 for the chosen strain) and can be cultured overnight to obtain counts. The D-optimal design allowed one to identify, among all the possible experiments, the subset leading to the best compromise between experimental effort and quality of information. The experimental matrix, made overall by 49 experiments, allowed us to estimate the coefficients of the model with good quality (the highest variance inflation factor, VIF, was 1.88) and with 30 degrees of freedom. The model explained 88.67% of the variance in fitting, with a standard deviation of the residuals of 0.12, and could be considered good enough for a qualitative analysis of the effects of factors and their interactions. When the model coefficients were

examined (Figure 4a), it was found that the total power of the two LEDs was by far the most relevant factor. Since the interaction and the quadratic term for factors X2 and X3 and the quadratic term for factor X5 were also quite relevant, the full interpretation of their effect could be exclusively obtained by looking at the response surface (Figure 4b). The isoresponse contours in Figure 4b–d, incorporating multiple curvilinear interactions display the combined effects of two experimental factors on the inactivation efficiency rate of the device. It is easy to understand that a total inactivation of the aerosolized bacteria (>99%) can be obtained with a total current of 200 mA or more when both LEDs are operative. Interestingly, LED2 seemed to have a much smaller effect. This could be due to the position within the device (bioaerosol flow always "sees" LED2 after LED1). The flow rate of the bioaerosol through the device was indeed another important factor that could significantly influence the UV-C inactivation efficiency, affecting the exposure time of microorganisms to UV-C light. The exposure time of the microorganisms to UV-C light is directly related to the flow rate. Higher flow rates result in shorter residence times within the device, which, in turn, leads to reduced exposure of the microorganisms to UV-C light. Even if the device performed equally well for any of the tested flow rates (12.5 to 50 L/min), an appropriate balance between flow rate and exposure time needs to be maintained to achieve a sufficient UV-C dose for an effective inactivation in real settings (much higher flow rates). Due to the limitations in modifying the experimental setup to directly investigate higher flow rates using one device and the objective of identifying ideal conditions for a potential future scalability study on the integration of multiple devices inside HVAC units or associated ductwork, an additional D-optimal design was conducted. In this second design, a reduced configuration (half of the original device) was used to mimic the reduction of exposure time in higher-flow HVAC units and tested together with the significant factors identified in the first design. The variable X2 (particle size) was retained to confirm the fact that it was not significant in the studied range. The other significant terms in the model were, as expected, the linear effects of X3 (LED power) and X4 (airflow), as well as the quadratic term for X4. By looking at the contour plots in Figure 5b and 5c, it is clear that a significant improvement can be obtained when a "double" configuration is used, as this increases the residence time inside the device. With lower flows and the device in "single" mode, it was possible to achieve no more than 80% inactivation, even with higher LED power.

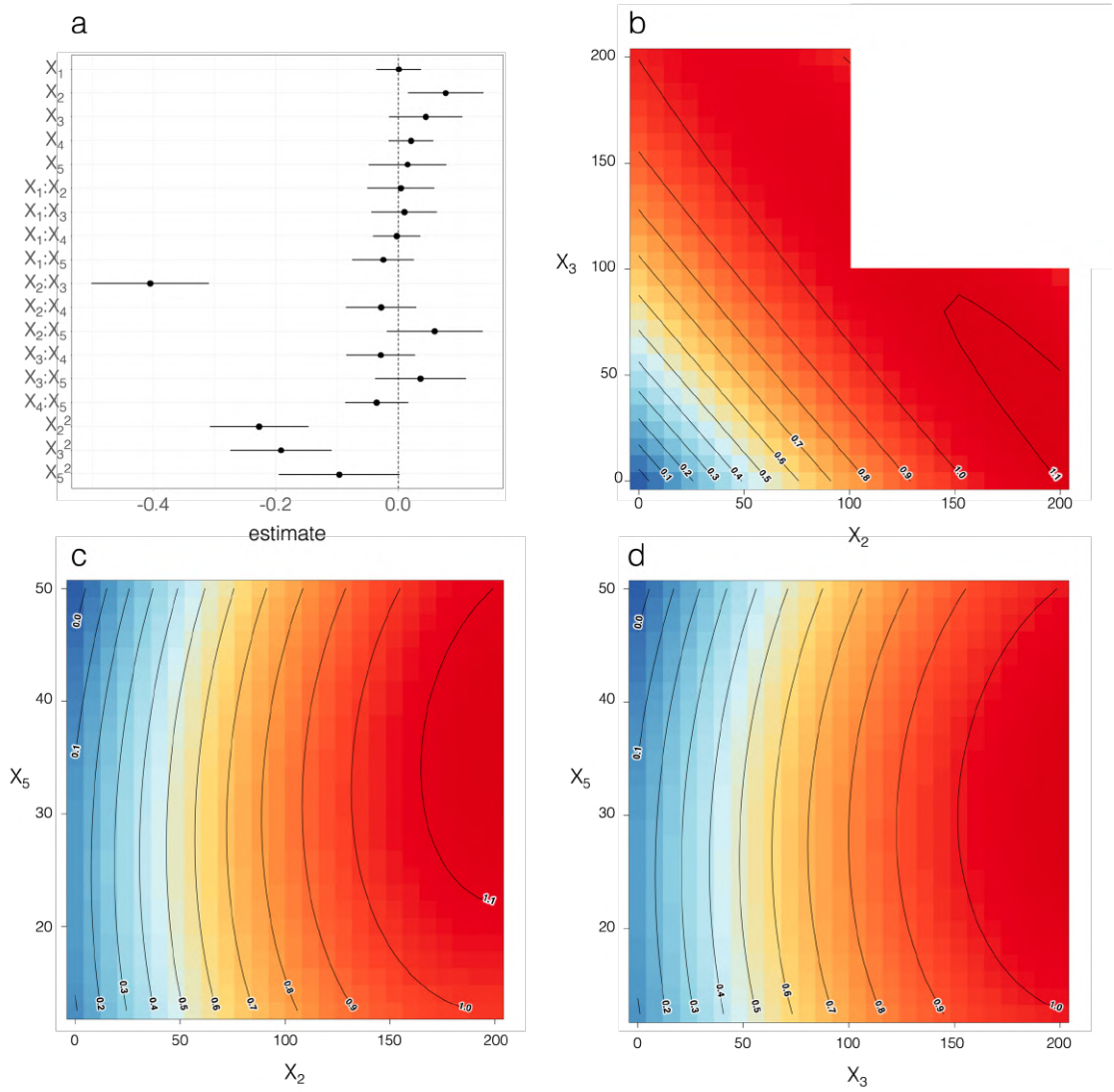

Figure 14. (a) Plot of the model coefficients with bars indicating the 95% confidence intervals. The statistically significant terms are the linear term X_2 ($p = 0.01513$), the interaction X_2:X_3 ($p < 0.001$), and the quadratic terms for X_2 ($p < 0.001$), X_3 ($p < 0.001$), and X_5 ($p = 0.05587$). Factors are coded as Table2. (b) Response surface on the X_2:X_3 plane (power of LED1 vs power of LED2) for the inactivation efficiency rate (ε, %, contour curves). The levels for the other variables were set as 10^8 CFU/mL for X_1, 0.5 μm for X_4, and 50 L/min for X_5. (c) Response surface on the X_2:X_5 plane (power of LED1 vs airflow) for the inactivation efficiency rate (ε, %, contour curves). The levels for the other variables were set as 10^8 CFU/mL for X_1, 0 mA for X_3, and 0.5 μm for X_4. (d) Response surface on the X_3:X_5 plane (power of LED2 vs airflow) for the inactivation efficiency rate ε, %, contour curves). The levels for the other variables were set as 10^8 CFU/mL for X_1, 0 mA for X_2, and 0.5 μm for X_4.

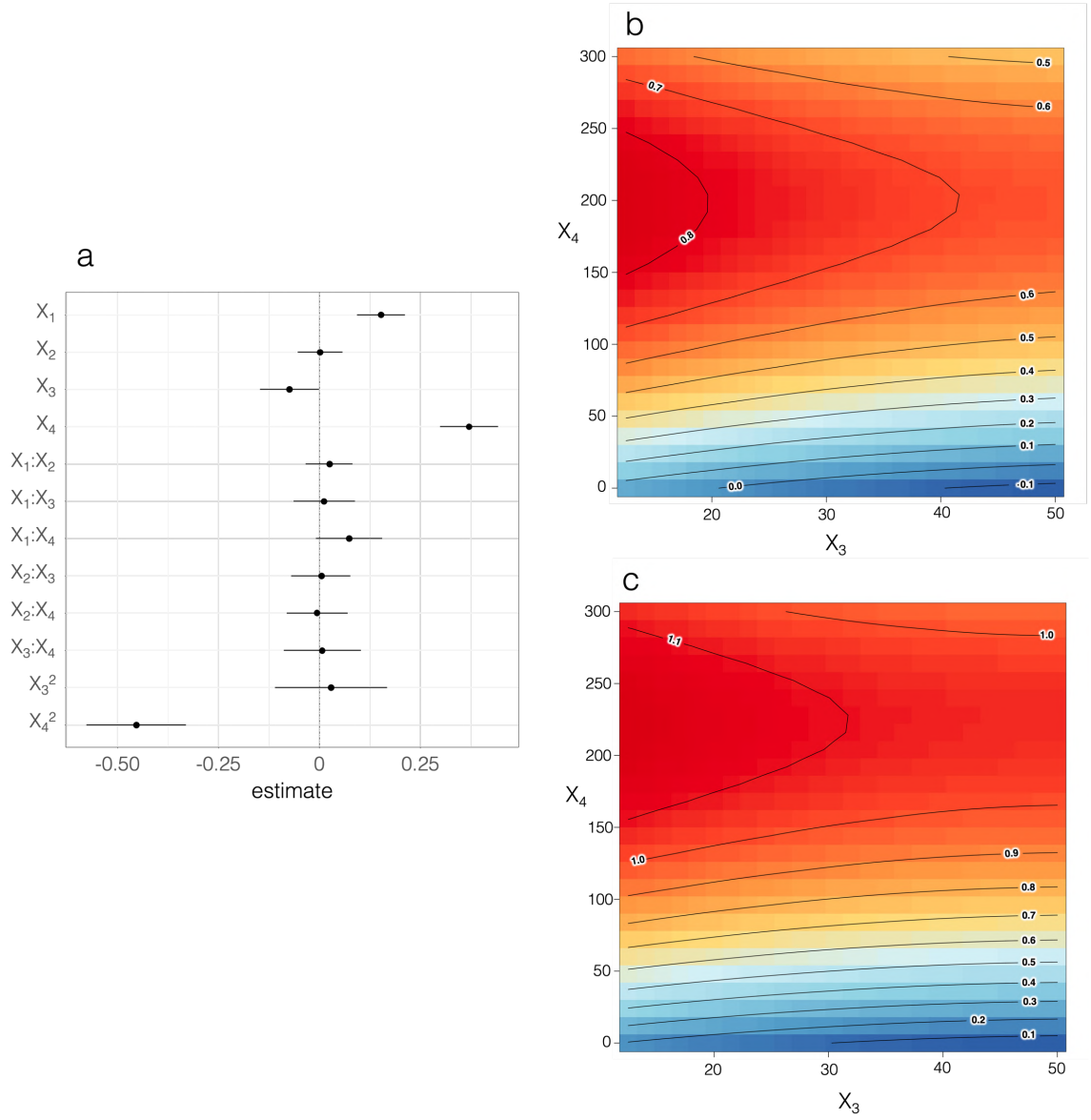

Figure 15. (a) Plot of the model coefficients with bars indicating the 95% confidence intervals. The statistically significant terms are the linear term for X_1 ($p < 0.001$), X_3 ($p = 0.04579$), and X_4 ($p < 0.001$), the interaction X_1:X_4 ($p = 0.07926$), and the quadratic terms for X_4 ($p < 0.001$). Factors are coded as in Table 3. (b) Response surface on the X_3:X_4 plane (airflow vs total LED power) for the inactivation efficiency rate (ε, %, contour curves) with single filter setup. The level for X_2 was set as 1.5 μm. (c) Response surface on the X_3:X_4 plane (airflow vs total LED power) for the inactivation efficiency rate (ε, %, contour curves) with double filter setup. The level for X_2 was set as 1.5 μm

3.3 Limitations

No performance test specifically targeting airborne bioaerosols was carried out. The composition and concentration of bioaerosols in natural air are complex and constantly fluctuating. Hence, it is challenging to utilize environmental bioaerosols in a controlled setting for evaluating the efficiency of a new device by using the culturing technique. Future studies employing real-time bioaerosol

monitoring technologies could complement this study by thoroughly assessing the effectiveness of the proposed device in field studies.

4 Conclusions

This study systematically and methodically explored the key parameters that impact the effectiveness of a novel UV-C LED aerodynamic device designed to diminish the viability of airborne pathogens without causing pressure loss. The inactivation efficiency of the device on a nonpathogenic strain of *E. coli* aerosolized in a confined environment was examined experimentally. Employing a comprehensive data-driven approach, this research successfully identified the essential power specifications, configurations, and airflow conditions necessary for optimal device performance under different scenarios. Moreover, we demonstrated that certain factors remain inconsequential across the entire range of investigations, which can yield significant practical insights. The findings clearly showed the importance of airflow in bioaerosol inactivation by an UV-C LED. This knowledge can be utilized to develop a rack or enclosure capable of accommodating various devices and additional components and facilitating their integration into heating, ventilation, and air conditioning (HVAC) systems in real-world applications.

5 Further developments

The experimental results obtained by this study have inspired an improved computer-aided design of the aerodynamic filter, published in *Applied sciences* [38].

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SECTION 3

Bioaerosol generation during dental procedures: quantification and identification studies at the dental clinic of the Maggiore Hospital in Trieste (Italy)

Paper in preparation

Abstract

Bioaerosols, consisting of airborne particles carrying biological materials such as bacteria, viruses, and fungi, represent a significant concern in healthcare settings due to their potential for infection transmission. Dental procedures, which generate high levels of aerosols, pose particular risks for both healthcare workers and patients. This study investigates the dynamics of bioaerosol deposition in the Maxillofacial Surgery and Dentistry Clinic at the Maggiore Hospital in Trieste (Italy), focusing on spatial and temporal variations during operational and non-operational hours, as well as microbial identification using the 16S rRNA metabarcoding technique.

Bioaerosol sampling was conducted using Petri dishes with Tryptic Soy Agar (TSA) placed at strategic locations within the dental clinic and the adjoining office as control. Samples were collected continuously and hourly over four days, with colony counts recorded post-incubation. A subset of colonies was identified via polymerase chain reaction (PCR) amplification of the 16S rRNA gene, followed by analysis for taxonomic classification. Data were analyzed for spatial and temporal trends, with correlation analysis performed using modeFRONTIER software.

The results demonstrate how bioaerosol concentrations were highest near the patient and decreased with distance, reflecting a proximity-dependent gradient. Colony counts were significantly higher during operational hours compared to downtime, with over 90% reduction in deposition rates observed after clinic operations ceased. Unexpectedly, control samples from the adjoining office exhibited elevated colony counts, suggesting external factors influencing bioaerosol deposition. Taxonomic analysis revealed that all identified colonies belonged to the genus *Staphylococcus*, including opportunistic pathogens such as *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*.

These findings highlight the critical role of spatial dynamics, ventilation, and procedural activities in bioaerosol dispersion. By elucidating the dynamics of bioaerosol generation and deposition, the findings support the need for targeted interventions, such as enhanced air filtration and strategic clinic design, to mitigate bioaerosol risks.

1 Introduction

Bioaerosols, composed of airborne particles carrying biological materials like bacteria, viruses, and fungi, are a major concern in healthcare environments due to their potential for transmitting infections. This issue is particularly pronounced in dental clinics, where aerosol-generating procedures contribute significantly to the risk of airborne contamination. Oral health care is widely recognized as a high-risk category of occupations associated with aerosol production, as oral cavity harbors microorganisms that can pose a risk for cross-contamination and infection for both dental healthcare workers and their patients [1].

The oral microflora comprise commensal, symbiotic, and in some cases pathogenic microorganisms. It plays a pivotal role in maintaining oral and systemic health by preventing pathogenic colonization and supporting metabolic functions. Variation to this equilibrium can trigger oral diseases like caries and periodontitis and have been associated with broader systemic health conditions, such as cardiovascular disease and diabetes. Advanced in recent techniques such as the high-throughput whole-genome sequencing have revealed the complexity of this microbiome, identifying up to 1,179 microbial taxa within the oral cavity [2].

Dental aerosols are generated during a range of procedures, from routine cleanings and fillings to complex dental surgeries, with high-speed tools such as ultrasonic scalers, air polishers, and air/water syringes contributing significantly due to their ability to create a fine mist by rapidly mixing air, water, and bodily fluids [3]. In particular, according to the World Health Organization, aerosol-generating procedures in oral healthcare include those involving spray-generating equipment, such as ultrasonic scaling, polishing, and periodontal treatments, as well as dental preparation with high- or low-speed handpieces, crown or bridge cementation, mechanical endodontic treatment, and surgical tooth extractions or implant placements [4]. These procedures can aerosolize saliva, blood, and dental materials, dispersing microorganisms into the surrounding environment [1].

During dental treatments, the use of high-speed instruments and the "wet environment" created by saliva and water coolants generate a substantial spray that disperses in various forms [5]. The distribution of contaminated aerosol and spatter is highly variable and may be influenced by several factors, including the type of procedure he use of high-volume evacuation, the equipment employed, the pressure and flow rates of air or water, the position of the tooth in the mouth, the patient posture, and operator positioning and handedness [6]. Research by Polednik shows how aerosol and bioaerosol concentrations in dental clinics vary depending on the procedure, monitoring location, and measurement devices used [3].

Micik and colleagues conducted pioneering studies on dental environment bioaerosol [2,7,8]. They defined aerosols as particles smaller than 50 micrometers in diameter, capable of remaining airborne for extended periods before settling on surfaces or entering the respiratory tract. Smaller aerosol particles, ranging from 0.5 to 10 µm in diameter, can penetrate deeper into the lungs and are considered to pose the greatest risk for infection transmission. In contrast, *splatter*, i.e., particles larger than 50 µm, behaves ballistically, following a trajectory from the source until contacting a surface or falling to the floor. These larger particles remain airborne only briefly due to their larger size. The prevailing consensus is that aerosols under 50 µm pose the greatest risk in dentistry due to their prolonged airborne presence and ability to enter in the respiratory systems [9]. Therefore, infectious microorganism may be transmitted between patients and operators or from patient to patient through contaminated surfaces, materials or instruments used during dental procedures [7] (Figure 16). Since dental aerosols may contain opportunistic pathogens and create a hazardous environment, particularly for immunosuppressed patients, infection control precautions should be adopted to minimize any potential risk [6,8,9].

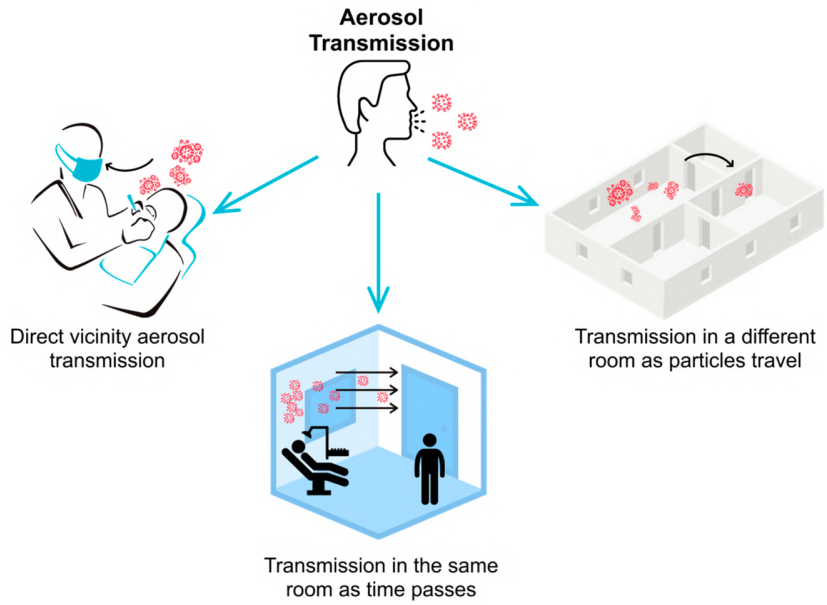

Figure 16. Aerosolized particles generated during dental operations can transmit infectious diseases via three paths: direct vicinity transmission; same room transmission as time passes; and different room or location transmission [10].

Dental bioaerosol concentrations rise steadily during treatment and gradually decrease after procedures are completed [6]. The microbial load and residence time of bioaerosols typically decay exponentially with travel time or droplet size. However, significant bioaerosol levels can still accumulate in enclosed spaces with limited ventilation [5]. So far, the related research on dental

clinics is also limited to examine bioaerosol temporal spatial distribution during whole oral cleaning surgery [7,11].

Numerous studies have been conducted to identify which dental procedures is linked with the highest levels of airborne bacterial aerosolization [12]. These studies commonly employ gravity sampling, a non-quantitative method where agar plates are exposed to collect airborne microorganisms by gravity [13]. This method tends to capture larger particles, potentially underrepresenting the smaller ones [14,15]. Despite its limitations, gravity sampling is considered reliable and reproducible, allowing for comparative analyses across multiple locations at the same time without disturbing the environment. However, gravitational settling method gives insights about the total number of the collected bioaerosol but it cannot be compared to the volume of sampled air [16].

However most studies use nonselective growth media, such as blood agar, to quantify total colony-forming units (CFUs) of airborne bacteria, though these methods do not distinguish eventual pathogenic species. For this purpose, some studies focused on the microbial composition rather than quantification of the bioaerosol in dentistry clinics, showing *Staphylococcus*, *Pseudomonas* and *Acinetobacter* genus as the most frequently identified [17,18]. Recent advancements in molecular techniques, including the 16S rRNA gene metabarcoding, have enabled more precise identification and quantification of microbial communities in bioaerosols, offering deeper insights into microbial diversity and shifts during clinical activities [19]. However, gravimetric settling methods, which utilize agar-based media, continue to serve as a fundamental approach for evaluating microbial deposition rates and monitoring environmental contamination within clinical settings.

The National Center for Chronic Disease Prevention and Health Promotion (CDC) has issued comprehensive guidelines to prevent infectious disease transmission and address occupational health concerns in dental settings [20]. Beyond standard precautions, additional measures may be required to mitigate the spread of diseases transmitted via airborne, droplet, or contact routes. The most effective strategy for preventing airborne transmission involves containing pathogens at their point of origin. Source-focused controls are particularly effective as they prevent the dispersion of aerosolized particles and droplet clouds. Additionally, advanced air treatment technologies, including ultraviolet (UV) radiation, ozonation, photocatalytic oxidation, and electrostatic precipitators, have been proposed to mitigate airborne contamination. Combining filtration and inactivation technologies may offer enhanced protection by targeting pathogens closer to their source [21–24].

This study aims to investigate the dynamics of bioaerosols in the dentistry clinic of Maggiore Hospital in Trieste (Italy), with a focus on their distribution and deposition during active treatment hours and subsequent downtime. This study also aims to identify the sampled bacteria by testing a protocol for

preliminary identification, evaluating its broader applicability and potential for future microbial and indoor quality research. By integrating molecular and culture-based approaches, the findings contribute to a broader understanding of bioaerosol behavior and its implications for infection control practices. The results of this study will support the development of a sanitizing technology, based on the filter tested in SECTION 2 of this thesis [24]. This technology, developed in collaboration with the Esteco company, aims to provide practical solutions for reducing bioaerosols during dental procedures and enhancing infection control in clinical settings.

2 Materials and methods

To assess the quantity and distribution of bioaerosols in the dentistry clinic, bioaerosol sampling was performed by placing Petri dishes filled with agar medium (Triptic Soy Agar – TSA) at seven sampling points. One Petri dish was placed on the lamp over the patient, i.e., the aerosol source; three Petri dishes were placed at three different distances from the patient (near the head at 0 meters, at 1 meter and at 2 meters); three additional Petri dishes were placed in the adjoining room (i.e., the office of the medical director) on the desk, on the bookshelf and near the window as control samples. For the samples at the three distances from the patient (i.e., 0 m, 1 m, and 2 m), two replicates were performed (called 0 m bis, 1 m bis, 2 m bis). ID samples description is summarized in Table 5.

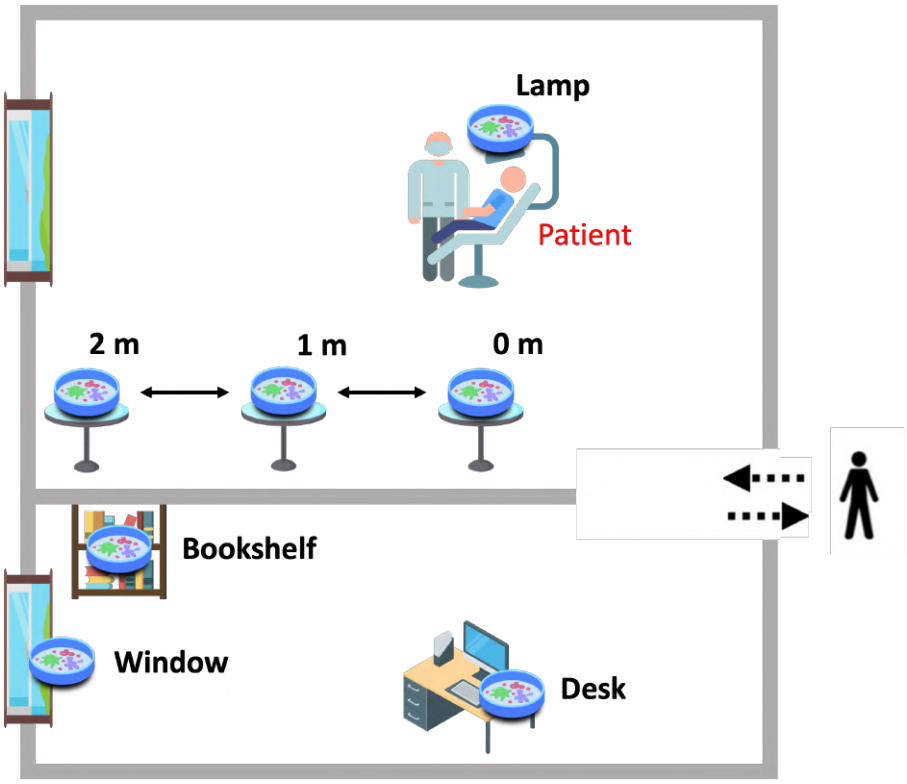

Figure 17. Sampling design in the dental unit at the Maggiore hospital of Trieste.

Table 5. ID sample description.

Sample ID	Sampling point	Room
Lamp	Plate placed over the lamp	Dentistry unit
0 m	Plate placed near the head of the patient, at 0 m	Dentistry unit
0 m bis (replicate)	Plate placed near the head of the patient, at 0 m	Dentistry unit
1 m	Plate placed at 1 m from the patient	Dentistry unit
1 m bis (replicate)	Plate placed at 1 m from the patient	Dentistry unit
2 m	Plate placed at 1 m from the patient	Dentistry unit
2 m bis (replicate)	Plate placed at 1 m from the patient	Dentistry unit
Window	Plate placed near the window	Dentistry unit
Bookshelf	Plate placed on the bookshelf	Adjoining office
Desk	Plate placed on the desk	Adjoining office

The sampling time started from 8 am to 2 pm, covering all the working time of the clinic. During this period of time, the bioaerosol particles were collected by passive collection on the agar plates. Gravimetric sampling was chosen as it allows for a simple and cost-effective method of sampling airborne particles over an extended period, without the need for complex equipment. Samples were then transported at the Department of Chemical and Pharmaceutical Sciences (DSCF, University of

Trieste) and incubated overnight at 37 °C. The day after the count of the grown colonies was performed in order to quantify the bioaerosol in the hospital environment.

2.1 Quantification and correlation analysis

Using quantity data, a scatterplot matrix was generated by modeFRONTIER software [25] (a multi-objective and multidisciplinary software that allows for statistical analysis), in order to analyse and visualize the relationships between each pair of variables in a dataset. modeFRONTIER was selected due to its robust statistical and visualization capabilities, which are particularly suitable for this type of multivariate analysis. Specifically, its ability to integrate scatterplots and histograms provides a comprehensive view of both data distributions and correlations, making it ideal for exploring patterns and relationships in complex datasets.

2.2 16S rRNA metabarcoding approach for identification analysis

The preliminary identification analysis of the sampled bioaerosol was performed following the metabarcoding of the 16S rRNA gene approach. The 16S rRNA gene is highly conserved across bacterial species, making it a reliable marker for bacterial identification while allowing differentiation at the genus or species level through its variable regions. The workflow of this analysis is shown in Figure 18.

Eight random colonies were picked (using an inoculation loop) from the agar plate present the higher microorganism contamination, i.e., the Lamp sample. The number of colony samples collected was chosen to ensure a manageable yet representative subset for a preliminary analysis, balancing practical constraints and the need for statistical relevance. Skipping the nucleic acid extraction step, each of the eight colony was mixed directly in a Polymerase Chain Reaction (PCR) mastermix and run following the PCR protocol described below. The broad-range bacterial primers 21M13_27F_YM (5'-AGAGTTTGATCMTGGCTCAG) and M13REV_1492R (5'-GGTTACCTTGTTACGACTT), which cover the entire V1-V9 region of the 16S rRNA gene, were used to amplify the bacterial community. Reactions were performed in a total volume of 15 µL reaction mix, composed of: picked colony, 7.5 µL of AccuStart II PCR SuperMix (QuantaBio), 0.45 µL of 27 Forward primer (10 µM), 0.45 µL of 1492 Reverse primer (10 µM), 0.75 µL of EvaGreen™ 20X (Biotium) dye, and 3.85 µL of DNase-free water. The following thermal cycles were used: 94° C for 3'; 94° C for 30''; 55° C for 15''; 72° C for 1.5'; 4° C for 5'. Real-time PCR reaction was performed on a CFX96 Touch Real-Time PCR Detection System on a C1000 Touch Chassis (Bio-

Rad, Hercules, CA, USA). A purification was performed on all samples using the Mag-Bind® Total Pure NGS kit (Omega Bio-tek Inc, Norcross, GA, USA), and each library was quality checked on agarose gel electrophoresis and quantified with Qubit™ Fluorometer (Thermo Fisher Scientific). The purified PCR products were sent to an external service to be sequenced on a Sanger platform (Eurofins Genomics, Ebersberg, Germany).

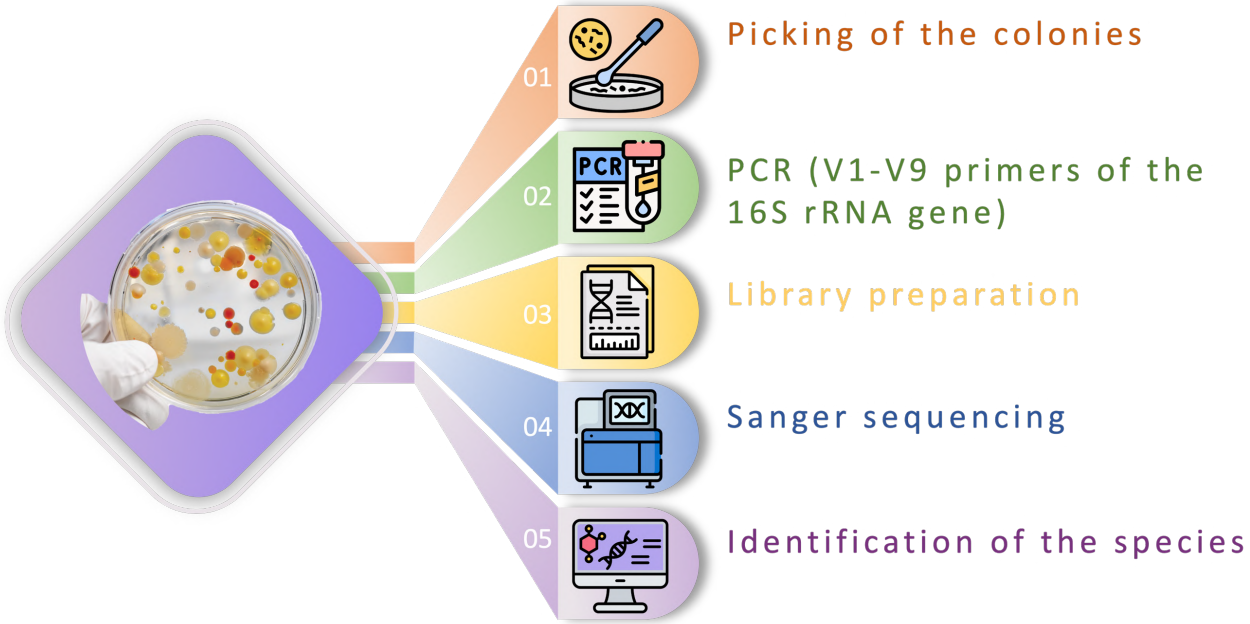

Figure 18. Workflow of the 16S rRNA metabarcoding analysis of the picked colonies grown on the Petri dish after the overnight incubation.

The chromatograms provided by sequencing facility representing each of our samples were manually cleaned at the 5' and 3' edges by removing low-quality regions and the primer sequences. This manual cleaning ensured the retention of high-quality sequence data for accurate downstream analysis. The clean sequences were classified by BLASTn search against the nt database [26]. The use of BLASTn ensures precise taxonomic classification by comparing the 16S rRNA gene sequences against a comprehensive database, with percentage identity serving as a key metric to validate the accuracy and confidence of the assignments. The results were evaluated as valid hits if the e-value was lower than a threshold of $1x10^{-50}$. This threshold was chosen as it ensures a high level of confidence in the taxonomic assignments, minimizing the risk of false positives while maintaining an accurate classification. Best hits with a clear taxonomic assignment, determined by high sequence identity and a definitive taxonomic match in the database, were used for classification. In absence of clear taxonomic assignment of the best hit, the best hit with a clear taxonomic classification was used. In

a few cases multiple hits with the same e-value and identity were annotated as different species of a genus. In these cases, the taxonomy was assigned based on simple majority rule.

2.3 Deposition rate analysis of bioaerosol

At the Maxillofacial Surgery and Dentistry Clinic of Maggiore Hospital, Trieste, bioaerosol deposition was analyzed during routine dental procedures (8 am - 2 pm) and the subsequent downtime (2 pm - 6 pm). Petri dishes containing Tryptic Soy Agar (TSA) were strategically placed in two dental units (A and B) to passively collect bioaerosol particles by gravitational settling, for a total of four days of sampling. Sampling consisted of:

- two dishes exposed continuously from 8 am to 2 pm in active dental units;
- four additional dishes exposed hourly from 2 pm to 6 pm during clinic inactivity;
- a final dish exposed continuously for the entire study period (8 am - 6 pm).

After collection, samples were transported to the Department of Chemical and Pharmaceutical Sciences (University of Trieste) and incubated at 37 °C overnight. The day after the count of the grown colonies was performed. This approach quantified the bioaerosol deposition rate during operational and non-operational hours, providing insights into airborne contamination dynamics and assess the deposition rate of the bioaerosol in the dentistry clinic.

3 Results

3.1 Quantification and correlation analysis

Data were collected on ten days, with variables analyzed for each day including the number of patients, sampling hours, and bioaerosol measurements from seven different locations in the dentistry unit. These include the Window and four different distances from the patient (Lamp, 0 m, 1 m, and 2 m) in the operating room. The Bookshelf and the Desk in the adjoining office were assigned as control samples.

Table 6 provides a detailed overview of bioaerosol sampling data collected across sampling days. The variables include the number of patients present and the number of sampling hours for each day, alongside the colony counts measured at various sampling points. **Errore. L'origine riferimento non è stata trovata.** shows the mean values of the number of colonies for each sampling point,

calculated as the arithmetic average across all sampling days. The sampling point with the higher mean value is the Desk, with a mean number of colonies of 104.25, followed by the Lamp (101.92), 0 m (90.58), 1 m (76.41), and 2 m (72.8). In particular, the Desk samples, thought of as control samples, had the highest number of colonies overall, which is unexpected given their location distant from the patient. This result suggests potential external factors influencing bioaerosol levels at this site, which are discussed in the Discussion section. Except for the Desk samples, the quantification values generally increased as the Petri dishes were placed closer to the patient, highlighting a proximity-dependent gradient in bioaerosol levels. The lowest mean values, however, are attributed to the Window and Bookshelf samples, with mean colony counts of 30 and 38, respectively.

The data shows day-to-day variation in bioaerosol levels, likely influenced by surgical activity, environmental factors, and sampling duration. This dataset provides a robust basis for exploring spatial and temporal trends in bioaerosol dispersion and examining their associations with dentistry clinic operations and patient activity.

Table 6. Table containing information about daily bioaerosol sampling data: number of patients, hours of sampling, and colony counts of the samples (Desk, Bookshelf, Window, Lamp, 0 m, 1 m, 2 m).

Day	Number of patients	Number of hours	Desk colonies	Bookshelf colonies	Window colonies	Lamp colonies	0 m colonies	0 m bis colonies	1 m colonies	1 m bis colonies	2 m colonies	2 m bis colonies
05/02/2024	4	5	97	78	32	123	99	65	88	82	86	76
06/02/2024	3	3	77	27	15	58	50	60	44	33	59	46
07/02/2024	6	5	68	15	18	37	52	41	51	51	29	43
26/02/2024	5	6	95	24	19	117	112	91	76	88	74	76
27/02/2024	6	6	117	31	37	95	92	69	76	85	63	58
28/02/2024	4	5.5	129	45	37	130	113	113	95	93	100	89
18/03/2024	5	5	98	35	39	121	83	125	76	86	89	91
20/03/2024	5	6	88	43	39	106	90	82	79	79	78	73
25/03/2024	5	6	116	42	45	104	137	130	107	52	68	NC
27/03/2024	5	6	126	42	46	159	111	122	104	117	96	115
08/04/2024	6	6	107	40	15	112	97	96	77	82	71	80
10/04/2024	5	4.5	133	40	22	61	51	60	44	43	50	54

Table 7. Mean number of colonies in each sample in all the sampling days

Sample	Mean number of colonies
Desk	104.25
Lamp	101.92
0 m	90.58
0 m bis	87.83
1m	76.42
1 m bis	74.25
2 m bis	72.8
2 m	71.92
Bookshelf	38.5
Window	30.33

A scatterplot matrix was generated from all the variables using the modeFRONTIER software, providing a graphical representation of the relationships between each pair of variables in the dataset. It combines both histograms and scatter plots, providing a unique overview of the distributions and correlations of the dataset. The output is shown in Figure 19. The correlations for each pair of input variables are shown below the diagonal, with values ranging from -1 to +1. Values close to +1 indicate that the variables are highly directly correlated, while values close to -1 suggest strong inverse correlations. Conversely, values near zero reflect low or no correlation between the variables. Correlation coefficients are color-coded, ranging from blue (inversely correlated variables) to red (directly correlated variables). White color indicates no correlation between variables.

A high degree of correlation is observed between bioaerosol levels at Lamp, 0 m, and 1 m samples, with coefficients exceeding 0.9. Moderate correlations are observed between 2 m samples and those located closer to the patient, i.e., Lamp, 0 m and 1 m samples. Sampling points in the adjoining office (Bookshelf and Desk) exhibit weak correlations with clinic sampling points (coefficient values < 0.4). The Number of patients correlates moderately (e.g., 0.656 with the Hours variable), reflecting expected increases in bioaerosol levels with a higher patient throughput and prolonged activity.

Histograms suggest unimodal distributions for most variables, with some variability likely due to fluctuations in activity, such as varying numbers of patients or procedural intensity, or environmental factors like changes in ventilation or ambient airflow.

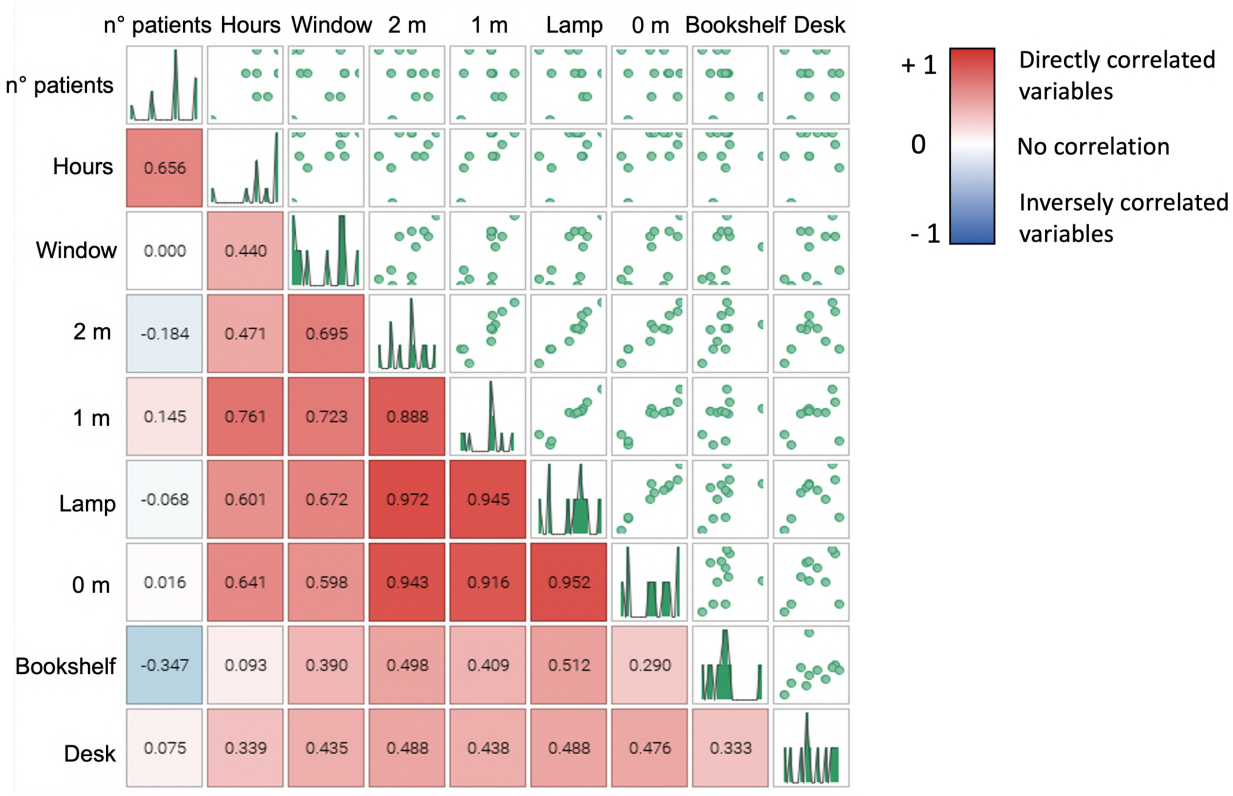

Figure 19. Scatterplot matrix generated using modeFRONTIER software, showing the relationships and correlation between bioaerosol sampling variables, including number of patients, sampling hours, and colony counts at various sampling points: Window, 2 m, 1 m, Lamp, 0 m, Bookshelf, and Desk. The correlations for each pair of input variables are shown below the diagonal, with values ranging from -1 to +1; values close to +1 show that the variables are highly directly correlated, while values close to -1 indicate that the variables are highly inversely correlated. Histograms on the diagonal represent the distribution of individual variables, while off-diagonal scatterplots and correlation coefficients illustrate pairwise relationships, with color coding indicating the strength and direction of the correlations (blue: negative, red: positive, white: no correlation).

3.2 16S rRNA metabarcoding approach for identification analysis

Using the metabarcoding of the 16S rRNA gene approach, several species were identified from colonies of the Lamp sample. Table 8 shows the best taxonomic hits for each colony based on BLASTn results, along with their percentage identity, providing insight into the microbial composition of the analyzed bioaerosol sample. In particular, this approach highlights the reliability of the method in identifying species with high fidelity. All picked colonies belongs to the same genus *Staphylococcus*. In particular, the species of *Staphylococcus* genus found in the samples are *S. epidermidis*, *S. haemolyticus*, *S. cohnii*, *S. hominis*, *S. xylosus*, and *S. saprophyticus*.

Table 8. Taxonomic identification and similarity percentages of the eight bacterial colonies picked from the Lamp sample. All samples had 0.0 as best hit e-value.

Colony	Best hit species	Best hit % identity

Colony 1	*Staphylococcus epidermidis*	99.75%
Colony 2	*Staphylococcus haemolyticus*	99.83%
Colony 3	*Staphilococcus cohnii*	99.72%
Colony 4	*Staphylococcus haemolyticus*	99.75%
Colony 5	*Staphylococcus hominis*	99.67%
Colony 6	*Staphylococcus haemolyticus*	99.56%
Colony 7	*Staphylococcus xylosus*	99.65%
Colony 8	*Staphylococcus saprohyticus*	98.72%

3.3 Deposition rate analysis of bioaerosol

Data were collected on four days of sampling (10/06/2024, 11/06/2024, 25/11/2024, and 02/12/2024). Bioaerosol colony counts (Table 9) were consistently higher during the whole-day samples (8 am – 6 pm), with a mean colony count of 170.6. This was followed by those detected during clinic operational hours (8 am – 2 pm), which showed a mean value of 145.63, indicating significant bioaerosol activity driven by routine dental procedures during operational periods (Table 10).

Dental Unit A showed moderate activity, with counts ranging from 105 to 147 colonies during active hours, while Dental Unit B displayed consistently higher deposition rates, with colony counts between 158 and 193 colonies.

A marked reduction in colony counts was observed after clinic operations ceased, as hourly sampling revealed a consistent decline, with the lowest counts recorded between 5 pm to 6 pm. On 10/06/2024, Dentistry Unit A showed a significant decrease in colony counts, dropping from 16 colonies (2 pm – 3 pm) to one colony (3 pm – 4 pm), exemplifying the rapid settling of bioaerosols during inactive periods. Similar trends were observed on other days, with counts in both units stabilizing at minimal levels (0-8 colonies) by 5 pm to 6 pm.

Cumulative counts (8 am – 6 pm) for each day show a clear difference between the two dentistry units, as the unit B reached higher totals compared to the unit A, highlighting a more significant bioaerosol burden.

Table 9. Bioaerosol colony counts across operational and inactive hours in Dental Units A and B. This table reports the colony counts recorded during operational hours (8 am – 2 pm), subsequent hourly intervals (2 pm – 3 pm, 3 pm – 4 pm, 4 pm – 5 pm, 5 pm – 6 pm),

and full sampling period (8 am – 6 pm) for two dental units (A and B) on four sampling days (10/06/2024, 11/06/2024, 25/11/2024, 02/12/2024).

		8 am – 2 pm colonies	2 pm – 3 pm colonies	3 pm – 4 pm colonies	4 pm – 5 pm colonies	5 pm – 6 pm colonies	8 am – 6 pm colonies
10/06/2024	**Dental unit A**	147	16	1	4	3	171
10/06/2024	**Dental unit B**	193	11	0	2	2	208
11/06/2024	**Dental unit A**	140	8	14	1	3	155
11/06/2024	**Dental unit B**	120	16	8	3	12	168
25/11/2024	**Dental unit A**	105	16	3	5	2	131
25/11/2024	**Dental unit B**	158	21	2	8	1	208
02/12/2024	**Dental unit A**	127	8	3	2	2	134
02/12/2024	**Dental unit B**	175	6	1	5	3	190

The percentage decrease in bioaerosol deposition rates highlights the dynamics of airborne contamination (Table 10). Hourly declines between 2 pm – 3 pm and 5 pm – 6 pm show an average reduction of > 90%, emphasizing the reduced microbial load in inactive periods.

Table 10. Mean bioaerosol colony counts and average percentage decrease during operational and inactive periods. This table summarizes the mean colony counts across different sampling hours (8 am – 6 pm) and the corresponding average percentage decrease in bioaerosol deposition during inactive periods. The data highlight the decline in microbial load over time, emphasizing the reduced airborne contamination after clinic operations cease.

Sampling hours	Mean number of colonies	Average % decrease
8 am – 2 pm	145.63	/
2 pm – 3 pm	12.75	-91%
3 pm – 4 pm	4	-97%
4 pm – 5 pm	3.75	-97%
5 pm – 6 pm	3.5	-98%
8 am – 6 pm	170.63	/

4 Discussion

4.1 Quantification and correlation analysis

Abundance data by colony counts show that the sample with the highest mean value is the Desk, with a mean number of colonies of 104.25. Initially considered a control point, the Desk was assumed to have limited bioaerosol exposure. However, its high colony count challenges this assumption, indicating that environmental and behavioral factors, such as common office activity and surface contamination, play a significant role in bioaerosol accumulation. Additionally, this discrepancy could be explained by the open nature of the office space, where increased human activity without personal protective equipment contributes to greater bioaerosol emissions. These findings highlight the potential for environmental factors to create bioaerosol hotspots, even in zones assumed to be less exposed to direct emissions.

Following the Desk, the Lamp (101.92), 0 m (90.58), 1 m, and 2 m (72.8) samples exhibited progressively lower mean colony counts, highlighting a gradient of dispersion and dilution of the bioaerosol. The gradient reflects the dynamics of bioaerosol movement, where concentrations decrease as distance from the source increases. This may be due to natural settling, airflow patterns, and dilution effects. These findings have significant implications for dentistry clinical practice, suggesting that possible bioaerosol mitigation strategies should focus on areas close to the patient to effectively reduce exposure risks.

The lowest mean values, however, are attributed to the Window and Bookshelf samples, with mean colony counts of 30 and 38, respectively. This may be due to these locations being further from the primary bioaerosol source (i.e., the patient for the Window sample and people walking within the dentistry clinic for the Bookshelf sample) and potentially experiencing less human interaction or environmental disturbance compared to other sampling points. As a result, fewer emitted particles reach these locations. These observations underscore the importance of spatial placement in influencing bioaerosol dispersion and deposition.

The high degree of correlation between Lamp, 0 m and 1 m suggests that these sampling points, located in proximity of the patient, capture similar bioaerosol dispersion patterns. It is important to notice that these samples are characterized by the higher bioaerosol concentration in the dentistry unit, due to the proximity to the bioaerosol source, i.e., the patient.

The moderate correlation between 2 m and Lamp, 0 m and 1 m samples indicates a diminishing bioaerosol concentration gradient. The scatterplot matrix reveals a pronounced proximity effect, where bioaerosol concentrations are highest near the patient and gradually decrease with distance.

This is supported by the strong positive correlations (coefficients > 0.9) observed among the sampling points located within the immediate vicinity of the patient: Lamp, 0 m, and 1 m. These findings align with the expected behavior of bioaerosol particles, which are typically emitted from human activity (e.g., breathing, speaking, coughing, or dental procedures using high-speed dental tools) and exhibit high concentrations near the source. The reduction reflects the natural settling or dispersion of bioaerosol particles due to gravitational forces and airflow dynamics. The behavior of bioaerosol particles, particularly their settling velocities and resultant concentration gradients, is influenced by their size. Larger particles, which often carry bacteria, tend to settle more rapidly due to gravitational forces, leading to higher concentrations near the emission source [27]. This highlights the relationship between particle size and deposition rates. As the present study was conducted using gravitational settling method for the collection of the bioaerosol, dominant larger particles were sampled, leading to deposition near the source, as reported.

The weak correlations between office, i.e., Bookshelf and Desk, and clinical samples indicate effective separation of the clinical environment from the adjoining office, likely due to airflow barriers and minimal source emissions. However, further evaluation is needed to determine whether this separation is sufficient to ensure overall safety and minimize cross-contamination risks, particularly under varying operational conditions or increased patient activity. This can be achieved by assessing whether the microbiome profiles of the samples from the two rooms are consistent and by determining if pathogenic microorganisms associated with dentistry procedures are present in the Desk and Bookshelf samples (e.g., by assessing microbiological markers). This reinforces the need for proper clinic layout design to mitigate cross-contamination.

In addition, the impact of the dentistry activity (number of patients) on control areas (i.e., the office room) is minimal, as correlations with office samples are low (<0.3). The spatial concentration gradient observed in this study may be influenced by airflow patterns. Insufficient ventilation near the patient area could exacerbate bioaerosol accumulation, whereas air movement in the adjoining office likely disperses particles, reducing correlations between the two zones.

The moderate correlation between patient numbers and bioaerosol levels in clinic samples highlights the operational dependency of bioaerosol load, suggesting that scheduling and patient throughput directly influence contamination risk. For instance, days with higher patient numbers may result in elevated bioaerosol concentrations due to prolonged procedures or more frequent use of dental instrumentation. Implementing alternating appointments or optimizing patient flow could help manage these risks more effectively. Furthermore, variability in bioaerosol generation is expected due to differences in operator techniques and manual handling of dental instrumentation.

This analysis underscores the critical role of spatial dynamics in bioaerosol management and highlights actionable recommendations such as improving ventilation systems, implementing targeted air filtration near high-risk zones, and enforcing stricter hygiene protocols in areas with high human activity. These measures can significantly enhance infection control in clinical environments and minimize contamination risks.

4.2 16S rRNA metabarcoding approach for identification analysis

Oral microflora, the ecological community of oral commensal, comprehends symbiotic, and pathogenic microorganisms, is the second largest microbial community in the human microbiome. High-throughput whole-genome sequencing and analysis have further revealed its complex diversity with up to 1,179 oral microbial taxa identified so far [28].

In the present study, all picked colonies from the Lamp sample belong to the same genus *Staphylococcus*, a group of Gram-positive, non-motile, facultative anaerobic bacteria. These bacteria are ubiquitous in the environment and can be detected in indoor spaces, such as hospitals and clinics, due to their presence on human skin and in respiratory secretions [29]. Their detection in bioaerosols pose a heightened risk due to prolonged air suspension and widespread dissemination, potentially exposing both patients and healthcare workers to pathogenic microorganisms.

All the identified species *S. epidermidis*, *S. haemolyticus*, *S. cohnii*, *S. hominis*, *S. xylosus*, and *S. saprophyticus* belong to coagulase-negative staphylococci (CoNS), a group of *Staphylococcus* species that lack the enzyme coagulase, distinguishing them from the more virulent *S. aureus* [30]. CoNS are generally considered less pathogenic but can act as opportunistic pathogens, particularly in immunocompromised individuals or patients with medical implants. They form an integral part of the normal human microbiota, primarily colonizing the skin, mucous membranes, and sometimes the gastrointestinal tract. Their ability to form biofilms makes them significant in healthcare-associated infections [31]. Their detection in dental clinic bioaerosols underscores their relevance as commensals, opportunistic pathogens, and environmental contaminants.

Several staphylococcal species, including *S. epidermidis*, *S. hominis*, and *S. cohnii*, are integral to the human microbiota [32,33]. The identification of these species in the dentistry unit highlights its role in indoor bioaerosols, likely originating from skin desquamation or respiratory droplets from patients, healthcare workers, or visitors. While they play crucial roles in maintaining skin homeostasis, they are also considered normal inhabitants of the oral cavity [34]. The species *S. epidermidis*, have been found in saliva samples from healthy individuals [35], while *S. haemolyticus* was found in isolates from dental patients [36].

This contributes to their presence in bioaerosols, particularly in environments with high human activity, such as dental clinics. The transition from commensal to pathogen is often facilitated by factors such as biofilm formation, immune suppression, or the presence of medical devices [37,38]. In fact, staphylococci are commonly associated with infections in clinical settings, often transitioning from commensals to opportunistic pathogens in immune-compromised patients [39]. For example, *S. haemolyticus* is linked to bacteremia, meningitis, skin and urinary tract infections, and even male genital dysfunction [40,41]. Its ability to form biofilms is a key virulence factor, enabling persistence on medical devices and contributing to infection chronicity [42].

S. saprophyticus is well-known for causing urinary tract infections, ranking second only to *Escherichia coli* in community-acquired cases. This highlights the diverse pathogenic potential within staphylococcal species, often influenced by specific host and environmental conditions.

The ability of staphylococci to persist in hospital environments is well-documented. *S. cohnii* and *S. hominis* species are commonly found on hospital surfaces, including floors, where they can persist for months [43,44]. These bacteria, often found alongside *S. epidermidis* and *S. haemolyticus*, are notable for their resilience and adaptability in nosocomial settings [45]. Their presence in bioaerosols reflects the microbial load of hospital environments and the potential for contamination.

S. xylosus, while less pathogenic, exemplifies the environmental adaptability of staphylococci. Commonly found in soils and on surfaces of food-processing plants, it is frequently introduced into clinical environments via human activity or environmental contamination [46,47]. The presence of *S. xylosus* in bioaerosols in a dental unit may originate from humans or environmental contamination. It may be introduced through skin shedding, respiratory droplets, or environmental dust. Although its detection is less concerning compared to highly pathogenic staphylococcal species, its ability to persist in diverse settings highlights the need for rigorous air and surface monitoring, especially in sensitive environments such as dental units.

The potential for zoonotic transmission among staphylococci is increasingly recognized [48,49]. This has significant implications for infection control in healthcare settings, as it highlights the need for enhanced surveillance and preventive measures to limit cross-species microbial transmission. Such measures include rigorous hygiene practices, proper handling of animals in healthcare environments, and regular monitoring of bioaerosols to identify potential zoonotic pathogens and mitigate their risks. *S. haemolyticus*, for example, has been isolated from both animals and humans, emphasizing the interconnectedness of human, animal, and environmental health. Strains have been isolated from both dogs and their owners, suggesting potential cross-species interactions [50].

The study of staphylococci in dental clinic bioaerosols provides valuable insights into their dual role as commensals and opportunistic pathogens. However, the absence of other genera might indicate methodological limitations, such as selective growth conditions. Understanding their ecological and clinical dynamics is crucial for developing strategies to mitigate their impact in healthcare and beyond.

4.3 Deposition rate analysis of bioaerosol

Despite slight day-to-day variations, a consistent pattern of elevated bioaerosol deposition during active hours and substantial reductions during downtime emerged across all four sampling days. This trend underscores the significant role of dentistry activities in influencing airborne contamination levels. Following clinic activity, bioaerosol counts decreased significantly, indicating settling of airborne particles [27]. In fact, across the four sampling days, the average reduction in colony counts between 2 pm and 6 pm reflected a predictable pattern of particle settling once dentistry activities ceased.

The significant difference between dentistry units (A and B) may reflect variations in dental procedures, number of patients, tools usage, or ventilation systems. Collecting data on these variables could help identify specific contributors to the observed differences and inform targeted interventions. Dentistry unit nit B consistently exhibited higher colony counts, suggesting it may have higher activity levels or less effective air circulation compared to Unit A. This elevated burden suggests a potential for higher exposure risks to both patients and staff.

There was a clear trend of decreasing bioaerosol deposition over time during downtime, with notable percentage decreases relative to the counts during active hours. This decrease highlights the reduced airborne contamination during non-operational hours, aligning with expectations of particle settling in the absence of activity [27]. This observation highlights the importance of scheduling downtime between procedures to allow for bioaerosol reduction to minimize contamination risks.

These results demonstrate a strong correlation between dental activity and bioaerosol deposition rates. The high colony counts during operational hours reflect the generation of bioaerosols from dental procedures, while the decline during downtime suggests that airborne particles settle over time when activities cease. The observed reductions during downtime highlight the effectiveness of passive settling in reducing airborne contamination. However, incorporating active ventilation or air purification systems could further minimize residual bioaerosols, particularly in high-activity units like Unit B. The persistent difference between the two units suggests the need to investigate

environmental factors such as air exchange rates, equipment-generated aerosols, and procedural variations.

5 Conclusions

By using simple passive sampling and counts, coupled with the 16S rRNA metabarcoding technique, this study highlights the presence of potentially hazardous bioaerosol generated during dental procedures, underscoring the risks posed to both healthcare workers and patients. This is particularly significant in the context of occupational health and infection control, where managing airborne microorganisms is critical to minimizing exposure to opportunistic pathogens and ensuring a safe clinical environment. Sampled colonies belong to the same genus *Staphylococcus*. These species are significant in both clinical and environmental contexts.

This study also demonstrates a strong correlation between dentistry activity and bioaerosol deposition rates for what concerns relatively coarse bacterial bioaerosol particles, prone to rapid settling. The high colony counts during operational hours reflect the generation of bioaerosols from dental procedures, while the decline during downtime suggests that airborne particles settle over time when activities cease.

These results underscore the importance of targeted interventions to mitigate bioaerosol risks in clinical settings. To address these risks effectively, a variety of mitigation strategies can be implemented, focusing on ventilation, filtration, and spatial design improvements. For instance, ventilation improvements, such as enhancing air exchange rates in high-exposure zones (e.g., near the patient and lamp), can significantly reduce local bioaerosol concentrations. Localized air filtration, such as deploying high-efficiency particulate air (HEPA) filters or advanced disinfection technologies, as the one mentioned in SECTION 2 of this thesis [24], could capture particles generated close to the patients prior to its dispersion in the indoor environment, enhancing the overall air quality in dentistry clinics. Spatial planning, such as strategic placement of equipment and barriers can further limit bioaerosol spread. For example, placing high-contamination equipment like ultrasonic scalers away from high-traffic areas and using barriers to segregate patient treatment zones can minimize bioaerosol diffusion. These strategies ensure that the proximity effect remains confined to safe levels and reduce the likelihood of cross-contamination within clinical spaces.

6 Further developments

Results from Section 2 and knowledge developed in Section 3 have driven to the development of a prototype of aspiration and UV-C filtration device to be integrated within dentistry units, for which testing is ongoing as in December 2024.

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SECTION 4

Bioaerosol sampling devices and pretreatment for bacterial characterization: theoretical differences and a field experience in a wastewater treatment plant

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Published on *Microorganisms*, http://dx.doi.org/10.3390/microorganisms12050965

Abstract

Studies on bioaerosol bacterial biodiversity have relevance in both ecological and health contexts, and molecular methods, such as 16S rRNA gene-based barcoded sequencing, provide efficient tools for the analysis of airborne bacterial communities. Standardized methods for sampling and analysis of bioaerosol DNA are lacking, thus hampering the comparison of results from studies implementing different devices and procedures. Three samplers that use gelatin filtration, swirling aerosol collection, and condensation growth tubes for collecting bioaerosol at an aeration tank of a wastewater treatment plant in Trieste (Italy) were used to determine the bacterial biodiversity. Wastewater samples were collected directly from the untreated sewage to obtain a true representation of the microbiological community present in the plant. Different samplers and collection media provide an indication of the different grades of biodiversity, with condensation growth tubes and DNA/RNA shield^{TM} capturing the richer bacterial genera. Overall, in terms of relative abundance, the air samples have a lower number of bacterial genera (64 OTUs) than the wastewater ones (75 OTUs). Using the metabarcoding approach to aerosol samples, we provide the first preliminary step toward the understanding of a significant diversity between different air sampling systems, enabling the scientific community to orient research towards the most informative sampling strategy.

1 Introduction

Bioaerosol is the aerosol containing components of biological origin that range in size from several nm to tens of μm [1]; it has relevant ecological [2,3] and health effects [4] when acting as a carrier of pathogens such as viruses, bacteria or allergens. The social impact of airborne pathogens is driving research and innovation to improve bioaerosol control and abatement technologies [5], including innovative photocatalytic systems and functional nanomaterials [6,7,8]. The air is a very complex matrix due to its dynamism, experiencing significant variation in the quantity, size, and composition of particles suspended within it. Given the variety of bioaerosols and the various ways in which they can interact, obtaining a representative sample is quite challenging. Additionally, the natural characteristic of the air matrix to dilute substances due to diffusion processes makes it difficult to capture specific microbiological events. The aim of the research heavily influences the selection of methods for collecting and analyzing samples. Different sampling devices can affect the results due to variations in their capability of separating particles by size, their efficiency, and the amount of sample they can collect. These differences can impact on the reliability of comparing results between studies, particularly if their objectives differ. Hence, there is a clear necessity for standardizing measurement procedures. Despite the wide array of research objectives, ensuring consistency within each approach is crucial for meaningful comparisons across different laboratories and experiments [9]. Culture-based approaches have been used traditionally to study bioaerosols; however, only a small portion of the overall microbial community is captured by culturing.

Molecular methods are thus applied for extending aerosol biodiversity characterization [10]. Currently, there are many different active bioaerosol sampling systems employed, with several others still in a development stage. Intercomparison studies have been published [11], considering eight different samplers and a commercial swirling aerosol sampler as a reference, in controlled laboratory situations. Among new devices, condensation growth tubes are promising ones, but they are still limitedly compared to other alternatives [12]. Some studies report on the performances of custom devices in both outdoor [13] and controlled settings [14]; other ones provide literature reviews without direct strict comparability in real settings [15]; and one study from a military research group provides qualitative indications on characteristics of devices [16]. An intercomparison was proposed considering sampling efficacy on aerosolized viruses [17] putting in strict proximity devices with highly different sampling flows, possibly generating interferences. A comparison between different sampling systems in the same outdoor environment can be helpful, and relevant bioaerosol sources provide significant scenarios to develop such experimental activities; wastewater treatment plants (WWTPs) [18] are considered among such sources, collecting microorganisms from excretions of

inhabitants of a certain area and treating sewage inputs by degradation of organic matter producing a small but not negligible generation of bioaerosols.

The sewage service collects, lifts, removes, and purifies the wastewater from basements of individual residences, industrial sites, urban centers, roadways, and public spaces. The gathered water is transported to the purification plant, where it is treated and returned to the environment. In a complex matrix like wastewater, an extensive variety of chemical and biological substances can be found, such as pharmaceuticals, exogenous contaminants [19], nutrient concentrations, and microorganisms [20]. These biological substances have been studied for decades, but mostly to monitor substance concentrations entering the wastewater treatment process or to monitor removal efficiencies of the WWTPs. It is interesting to note that the presence of various substances in wastewaters gives direct qualitative and quantitative information about the behavior of inhabitants within a certain wastewater catchment. In particular, wastewater represents the whole enteric pathogen load from a local catchment region and captures community disease burden, therefore providing an ideal matrix for disease monitoring and surveillance of human activity [21]. The first step in the process of treating urban wastewaters is the removal of the larger solid bodies thanks to a system of progressively finer grids which trap coarse objects like pieces of plastic, small branches, and leaves. Wastewater next passes into aeration tanks, where degritting takes place: a flow of air is introduced into the waters to raise oils to the surface, while also precipitating sands and loam to the bottom. According to some studies, the intense mixing and turbulence occurring in the aeration tank results in splashing, bubble bursting, and spraying, all of which contribute to the release of bioaerosols [22,23]. The intensity of this phenomenon is such that it poses even a health risk for WWTP employees, who are more prone than the general population to have a wide range of work-related diseases, such as respiratory and gastrointestinal ones [24,25]. Recently, these peculiar characteristics of wastewaters have been used as a surveillance technique called Wastewater-Based Epidemiology (WBE) [21]. WBE can be performed in many different ways, also using molecular biology techniques. One of these can be the environmental DNA (eDNA) metabarcoding, which is a technique that allows for the simultaneous identification of many taxa in the same environmental sample thanks to the analysis of short DNA sequences of one or few genes called DNA barcodes [26,27,28]. This technique can be used for many different matrices (i.e., air and water) and organisms; and, in particular, for prokaryotes, the 16S rRNA gene is used as barcode. This particular region is chosen for its evolutionary conservation, which allows for the use of a combination of conserved universal Polymerase Chain Reaction (PCR) primers flanking a short hypervariable region (V3 and V4), which will be amplified and sequenced by high-throughput Next-Generation Sequencing (NGS) [29]. The output of this process allows us to identify and determine the diversity of microorganisms thanks to reference libraries, which are

databases containing the DNA barcode assigned to previously identified taxa [30]. Overall, WBE based on eDNA metabarcoding is a non-invasive, efficient, cost-effective, and sensitive method which has the potential to supplement other approaches of biodiversity monitoring for ecological study and management on large spatial and temporal scales [31]. Even if the process of eDNA metabarcoding can produce accurately and inexpensively very large quantities of data, currently there is a lack of standardization and unification regarding bioaerosol sampling for molecular ecology research, mainly because the optimization process needed for each sampler use and the sample processing procedure significantly hinder the advance of bioaerosol science. Microorganisms in the air, unlike in other environmental matrices, such as water or soil, are present in the environment at low concentrations; therefore, it is crucial, first of all, to ensure that a sufficient quantity of genetic material is collected during the sampling phase in order to proceed with subsequent analyses. Additionally, the results of the research will also be influenced by the nucleic acid extraction protocol used, as it may have a greater or lesser recovery efficiency [32,33,34]. Due to these factors, it is difficult to compare different bioaerosol studies and, at the same time, understand how much any individual variable can influence the results [35]. The concentration, size distribution, and bacterial population of the bioaerosols that aerosolize from the wastewaters of WWTPs are not identical, and they depend on the different regions, the kind of wastewater treated, the season, the WWTP process selected, the technologies, and meteorological parameters [36,37,38,39]. In addition to the intrinsic factors of the WWTP, bioaerosol concentrations and compositions can be influenced by the equipment selection, the sampling time, and the adopted process [40]. Potential bacterial pathogens such as *Acinetobacter*, *Alcaligenes*, *Bacteroides*, *Chryseobacterium*, *Micrococcus*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas* have been detected in WWTP bioaerosols [41,42,43]. Other studies show WWTP bioaerosols affiliated with the phyla Proteobacteria, Bacteroidetes, and Firmicutes [40], while others identify heterotrophic and mesophilic bacteria belonging to genera *Pseudomonas*, *Micrococcus*, *Escherichia*, *Bacillus*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, *Mycobacterium*, *Acinetobacter*, *Actinomyces*, and *Clostridium* [44]. In one study conducted in Italy, geographically matching the present work, the most abundant genera of bacteria were *Bacillus*, *Acinetobacter*, and *Arcobacter* [45]. A detailed report of the dominant microbial communities detected in the bioaerosol of the WWTP environment was recently published by Singh and colleagues [46]. However, most of the studies found in the literature are based on traditional cultural methods, which can surely lead to a higher quantity of biomass necessary for the subsequent analyses, on the one hand; but, on the other hand, they can lead to an underestimation of the microbiological richness and biodiversity of the sampled community. Recent studies on WWTP bioaerosols characterized by 16S rRNA gene metabarcoding are compared in Supplementary Table

S1, showing how different choices in parameters for quantitation and experimental designs make results hardly comparable.

Following the 16S rRNA gene metabarcoding technique, we started with a characterization of the bacterial community present in the wastewaters of the WWTP of Trieste (Italy) to define a sampling strategy and laboratory protocols for the identification of the species present in the aeration tank of the WWTP, which is the aeration process expected to generate bioaerosol in significant quantities and on a constant basis [25,47]. Since there are no standards or guidelines which regulate outdoor bioaerosol sampling, with this study, we tested the performances and the sampling efficiency of three different commercial bioaerosol samplers that implement different aerosol collection principles and remarkable characteristics for the widespread use (swirling aerosol collection), ease of operability (gelatine filtration), and bioaerosol collection efficacy (condensation growth tubes). In particular, the present study focused also on the development of a procedure for the characterization of the microorganisms in the environmental bioaerosol by comparing three different sampling devices: the Airport MD8 (Sartorius, Goettingen, Germany), the BioSampler (SKC, Eighty Four, PA, USA), and the BioSpot-VIVAS (Aerosol Devices Inc., Ft. Collins, CO, USA).

2 Materials and methods

2.1 Bioaerosol samples

A total of 4 bioaerosol samples were collected as close as possible to the aeration tank emissions of the WWTP in Servola (Trieste, Italy) (i.e., 40 cm above the surface of the aeration tank water level, for which it must be added the height of each sampler that corresponds with the inlet of the devices) with three different sampling devices: Airport MD8 (sample ID: “MD8_tank”), BioSampler (sample ID: “SKC_PBS_tank”), BioSpot-VIVAS (samples ID: “Vivas_PS_tank”, and “Vivas_PBS_tank”). All samples were collected on the same day, starting collection simultaneously, except for “Vivas_PBS_tank”, which was added to obtain a hint on the relevance of type of bacterial collection medium (DNA/RNA shield™, as recommended by [12], vs. PBS, according to manufacturer’s recommendation) in aerosol sampling; DNA/RNA shieldTM could not be used with BioSampler due to generation of foam in the impinger, in addition to the costs related to a higher liquid collection volume for BioSampler (20 mL) vs. BioSpot-VIVAS (2.5 mL).

Since one of the aims of the study was to compare three different sampling devices for bioaerosol collection, an overall sampling volume of 1440 L was fixed for each sampler in accordance with the time of setting up, sampling, and availability of the structure. The instruments operated in field, being activated at the same time, with parameters set as summarized in Table 1.

Table 11. Summary of the experimental settings for each air sampling device.

	Airport MD8	BioSampler	BioSpot-VIVAS
Flow rate (L/min)	50	12.5	8
Total volume (L)	1440	1440	1440
Sampling Time	28.8 minutes	1.92 hours	3 hours

2.1.1 Airport MD8

The Airport MD8 is an air sampler used for the collection of microorganisms in indoor and outdoor environments. It is a sampling device that is easy to handle, portable, and quite small (300 mm × 135 mm × 165 mm for approximately 2.5 kg) and consists of a vacuum pump with timer and volumetric flow rate control. It can be used combined with gelatin membrane filters as an air filtration system, choosing the desired flow rate between four different values (10, 30, 40, and 50 L/min). This type of sampling system offers the possibility to solubilize the gelatin membrane filters in small liquid volumes (minimum 80–100 µL/cm2 filter area), allowing for further applications, such as microbial cultures and PCR [48] (see Table 2 for specifications). In the field, the gelatin filter was placed on the adapter of the sampler, using gloves and sterile tweezers. At the end of the sampling, the filter was placed in sterile Petri dishes sealed with parafilm and stored on ice until the arrival at the laboratory, where it was maintained at −20 °C. Before the DNA extraction, the filter was cut into pieces and placed in a clean tube, where sterile water was added in order to dissolve the gelatin. The tube was centrifugated for 10 min at 3000× g and then incubated at 37 °C for 10 min. The total DNA was isolated from the sample using the E.Z.N.A.® Soil DNA Kit (Cat# D5625, Omega Bio-tek Inc., Norcross, GA, USA). A blank DNA extraction was performed as control to guarantee the absence of any environmental contamination. After the DNA extraction, the sample was concentrated using the DNA Clean & Concentrator®-5 Kit (Cat# D4013, Zymo Research Corporation, Irvine, CA, USA) in 8 µL of elution buffer.

2.1.2 BioSampler

The BioSampler is a liquid-based impinger widely used for bioaerosol sampling [49]. It is a lightweight glass sampler, quite small but fragile, that consists in an inlet, through which airborne particles pass into the collection device, and an outlet connected to a high-volume suction pump (mod. Bravo BIO, TCR TECORA®). In the collection vessel, three tangential nozzles ensure a swirling motion of the collection liquid upward on the inner walls. A detailed scheme of the device is provided in [50]. This peculiar design minimizes re-aerosolization and bounce of particles, preserving the integrity and viability of microorganisms like viruses, bacteria, fungi, and molds [50]. According to manufacturer's recommendation, the BioSampler was operated at a flow rate of 12.5 L per minute (LPM), at which the cutoff size (50% efficiency) is about 300 nm [51] (see Table 2 for specifications). The collection liquid used was a phosphate-buffered saline solution (PBS), which was placed into the collection vessel for a total volume of 20 mL. Because of evaporation, at half of the sampling time, the collection vessel was refilled with PBS to 20 mL. Once the sampling finished, the PBS was transferred from the collection vessel into a fresh tube and stored on ice until the arrival at the laboratory. Here, the sample was concentrated by filtration on a 20 μm pore size cellulose filter, using a syringe. The filter was then placed in a sterile Petri dish, sealed with parafilm, and stored at −20 °C until the analyses. Before the extraction of the DNA, the filter was cut into little pieces and placed directly in the Distruptor Tube of the E.Z.N.A.® Soil DNA Kit (Cat# D5625, Omega Bio-tek Inc., Norcross, GA, USA). A blank DNA extraction was performed as the control to guarantee the absence of any environmental contamination. After the extraction of the DNA, the sample was concentrated using the DNA Clean & Concentrator®-5 Kit (Cat# D4013, Zymo Research Corporation, Irvine, CA, USA) in 8 μL of elution buffer.

2.1.3 BioSpot-VIVAS

The BioSpot-VIVAS is a bioaerosol sampler based on a condensation growth tube sampling method, which permits a gentle impingement for the wet collection of pre-concentrated air particles [52]. The flowrate of each growth tube is 1 L/min; therefore, the combined flowrate of all the eight growth tubes is 8 L/min, which is the total flowrate of the instrument in operation. A detailed scheme of the device is provided in [53]. Thanks to the overall low flowrate, which mimics the dynamics of particle deposition in human lungs, airborne particles are put under minimal stress when impacted onto the liquid surface of the collection solution, thus allowing for the sampling of viable microorganisms [53]. In particular, the BioSpot-VIVAS can sample a particle size range that varies from 5 nm to 10 μm, allowing for the sampling of ultrafine bioaerosol like viruses [54] (see Table 2 for specifications). The collection medium used was the DNA/RNA shield™, a preserving solution of nucleic acids, or

PBS, which placed into a sterile 35 mm × 11 mm Petri dish for a total volume of 2.5 mL. After the sampling, the DNA/RNA shield™ was transferred to a fresh tube and stored on ice until the arrival at the laboratory, where it was stored at −20 °C (sample id: "Vivas_PBS_tank" and "Vivas_PS_tank"). The PBS was instead concentrated by filtration on a 20 µm pore size cellulose filter using a syringe. The filter was then placed in a sterile Petri dish, sealed with parafilm, and stored at −20 °C. The sample stored in DNA/RNA shield™ was directly processed with the E.Z.N.A.® Soil DNA Kit (Cat# D5625, Omega Bio-tek Inc., Norcross, GA, USA), while the filtered sample was first cut into little pieces and then placed in the Distruptor Tube of the kit. After the extraction of the DNA, the sample stored in PBS was concentrated using the DNA Clean & Concentrator®-5 Kit (Cat# D4013, Zymo Research Corporation, Irvine, CA, USA) in 8 µL of elution buffer. A blank DNA extraction was performed as the control to guarantee the absence of any environmental contamination.

Table 12. Technical characteristics of the three air sampling devices: Airport MD8, BioSampler, and BioSpot-VIVAS.

	Airport MD8	**BioSampler**	**BioSpot-VIVAS**
Supplier	Sartorius (Goettingen, Germany)	SKC Inc. (Eighty Four, PA)	University of Florida (Gainesville, FL)
Sampling flow rate (L/min)	50	12.5	8
Sampling principle	Filtration	Swirling aerosol collection	Condensation growth tube
Support	Gelatine	Liquid	Liquid
Collection media	Gelatine	PBS	PBS, DNA/RNA shield™
Sampling volume	80 mm diameter	20 mL	2.5 mL
Weight	2.5 kg	0.16 kg *	24 kg
Dimensions	300 x 135 x 165 mm	220 x 50 x 50 mm *	760 x 485 x 370 mm

* Not including the high-volume suction pump needed for sampling.

2.2 Wastewater

In order to have a true representation of the microbiological community present in wastewater, in addition to bioaerosol samples, 11 water samples of 15 mL of volume were collected directly from the sewage. In particular, seven samples were collected by grab sampling for each day (sample IDs: "Water_1", "Water_2", "Water_3", "Water_4", "Water_5", "Water_6", and "Water_7"), while the other four were the result of a composite process of sampling of 24 h in the same days, for which a

small amount of water was sampled every hour over the course of the 24 h and stored in a single tank (sample IDs: “Water_24h_1”, “Water_24h_2”, “Water_24h_3”, and “Water_24h_4”). See Table 3 for the experimental design. Once in the laboratory, water samples were concentrated on a 20 µm pore size cellulose filter, using a syringe. The filter was then placed in a sterile Petri dish, sealed with parafilm, and stored at −20 °C until the analyses. For the extraction of the total DNA, the filter was first cut into little pieces and then placed directly in the Distruptor Tube, following the protocol of the E.Z.N.A.® Soil DNA Kit (Cat# D5625, Omega Bio-tek Inc., Norcross, GA, USA). A blank DNA extraction was performed as the control to guarantee the absence of any environmental contamination.

Table 13. Experimental design of the samples collected during the study.

Wastewater	**Sample ID**	**Sampling**		**Day**
	Water_24h_1	Composite		2
	Water_24h_2	Composite		3
	Water_24h_3	Composite		5
	Water_24h_4	Composite		6
	Water_1	Grab		1
	Water_2	Grab		2
	Water_3	Grab		3
	Water_4	Grab		4
	Water_5	Grab		5
	Water_6	Grab		6
	Water_7	Grab		7
Aerosol	**Sample ID**	**Sampler**	**Support medium**	**Day**
	MD8_tank	Airport MD8	Gelatine filter	6
	SKC_PBS_tank	BioSampler	PBS	6
	Vivas_PS_tank	BioSpot-VIVAS	DNA/RNA shield™	6
	Vivas_PBS_tank	BioSpot-VIVAS	PBS	8

2.3 qPCR, library preparation, and sequencing

After the DNA isolation, a quantitative real-time PCR reaction was performed on a CFX96 Touch Real-Time PCR Detection System on a C1000 Touch Chassis (Bio-Rad, Hercules, CA, USA). The isolated DNA was used as a template to amplify the V3–V4 hypervariable region of the 16S rRNA gene, using three PCR primers, in order to reduce amplification biases: 515 Forward 5′-GTGYCAGCMGCCGCGGTAA-3′ [55], 806 Reverse 5′-GGACTACNVGGGTWTCTAAT-3′ [55], and 802 Reverse 5′-TACNVGGGTATCTAATCC-3′ [56]. PCR conditions were identical for all

samples. Reactions were performed in a total volume of 15 µL reaction mix, composed of 2 µL of DNA template, 7.5 µL of AccuStart II PCR SuperMix (QuantaBio, Beverly, MA, USA), 0.6 µL of 515 Forward primer (10 µM), 0.3 µL of 802 Reverse primer (10 µM), 0.3 µL of 806 Reverse primer (10 µM), 0.75 µL of EvaGreen™ 20× (Biotium, Fremont, CA, USA) dye, and 3.55 µL of DNase-free water. The following thermal cycles were used: 94 °C for 3′, 94 °C for 20″, 55 °C for 30″, and 72 °C for 1′. The second PCR amplification was performed in a total reaction volume of 25 µL, containing the same reagents as the first one, but adding 1.5 µL barcoded/TrP1 (10 µM) as the primer and 1 µL of the first PCR amplification as the template. The following conditions were used: 94 °C for 3′, 94 °C for 10″, 60 °C for 10″, and 72 °C for 30″. A purification was performed for all the samples using the Mag-Bind® Total Pure NGS kit (Omega Bio-tek Inc., Norcross, GA, USA), and each library was quality checked on agarose gel electrophoresis and quantified with Qubit™ Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Finally, libraries were pooled in equimolar amounts and sequenced on an Ion Torrent PGM System.

2.4 Bioinformatic analysis

The raw reads obtained within the frame of this study were imported into the CLC Genomics Workbench environment (Quiagen, Hilden, Germany). The quality of raw reads was evaluated with the Quality Control (QC) for sequencing reads tool to have a broad perspective of the sequencing process result and to optimize the subsequent trimming steps. The trimming operation was performed to increase the quality of the reads, and a QC of the trimmed reads was generated to evaluate the efficiency of the trimming process. On trimmed reads, an operational taxonomic unit (OTU) clustering was performed using the SILVA SSU 99% (v138.1) as reference database [57], with a similarity threshold of 97%. OTUs were then aggregated at genus level, and a filter selection was performed with the following parameters: “ID does not contain N/A”, “combined abundance ≥ 100”, and “taxonomy does not contain chloroplast”. The OTU table was exported, and the relative abundance of bacterial genera and the alpha diversity rarefaction curves were obtained and plotted within a Python environment.

3 Results

The sequencing process provided 129167 sequences (available at NCBI under the bioproject ID PRJNA1083383), with a median length of 250 bp. A total of 86 188 reads with an average length of 250 bp were kept after quality filtering, and their clustering resulted in 80 OTUs. A total of 70 087 trimmed reads and 75 OTUs were obtained from water samples, and 16 101 of trimmed reads and 64 OTUs were obtained from air samples (Supplementary Materials Table S2). In the aerosol samples, the bacterial groups were all dominated by the following bacteria OTUs: *Stenotrophomonas* (64.24% of total sequences in all aerosol samples), *Klebsiella* (14.92%), *Enterobacter* (4.54%), *Acinetobacter* (3.13%), *Delftia* (2.85%), and *Pseudomonas* (2.62%). In the wastewater samples, the most abundant genera of bacteria were *Acinetobacter* (26.37% of total sequences in all water samples), *Arcobacter* (17.44%), Arcobacteraceae (13.7%), *Pseudarcobacter* (5.19%), *Bacteroides* (4.94%), and *Aeromonas* (4.92%).

There is clear evidence of a difference in bacterial biodiversity between aerosol and wastewater samples: in wastewaters, an unambiguous pattern with a comparable biodiversity is observed between all the samples collected, while for bioaerosol, a certain degree of genus-level abundance variability appeared in relation to the different sampler and mediums (Figure 1 and Supplementary Figure S1). In particular, among the air samples, the "Vivas_PS_tank" contains a larger number of genera compared to the others, which instead displayed a striking dominance of bacteria of genus *Stenotrophomonas*. The most abundant genera found in this sample are *Klebsiella*, *Enterobacter*, *Pseudomonas*, and *Acinetobacter*. A significant difference can also be noticed between the two samples collected both by the BioSpot-VIVAS, but only differing for the collection medium ("Vivas_PBS_tank", with the PBS as collection medium, and "Vivas_PS_tank", with the DNA/RNA shield™ as collection medium). Also, in this case, the sample collected on the DNA/RNA shield™ medium shows a larger number of genera. Among the wastewater samples, the grab and the composite samples have comparable abundances of bacterial genera.

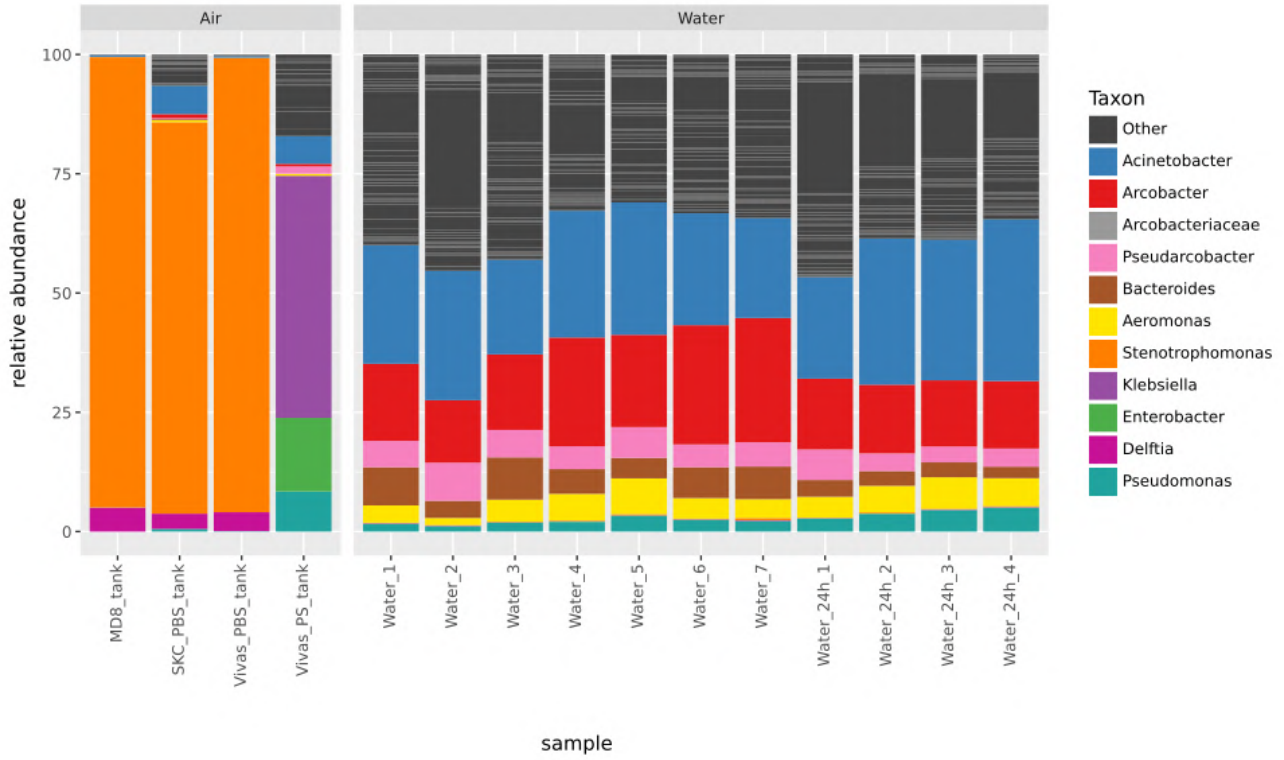

Figure 20. Relative abundance barplots of most dominant OTUs detected in air and wastewaters samples of the aeration tank of the WWTP of Servola (Trieste). Genera of the six most abundant OTUs in air and wastewater are color coded, and dark gray codes are for all genera that were grouped under the "Other" category. The air sample collected using the Airport MD8 has the sample ID "MD8_tank". The air sample collected using the BioSampler has the sample ID "SKC_PBS_tank". The air samples collected using the BioSpot-VIVAS have the sample IDs "Vivas_PBS_tank" (sampled using the PBS as collection media) and "Vivas_PS_tank" (sampled using the preserving solution of nucleic acids DNA/RNA shield™ as collection media). The wastewater samples that were collected by grab sampling for each day have the sample IDs "Water_1", "Water_2", "Water_3", "Water_4", "Water_5", "Water_6", and "Water_7". The wastewater samples that were the result of a composite process of sampling of 24 h in the same days have the sample IDs "Water_24h_1", "Water_24h_2", "Water_24h_3", and "Water_24h_4".

The difference in community compositions between air and wastewater samples is further supported by the alpha diversity rarefaction curves of the bacterial genus richness (Figure 2). The air samples show a smaller amount of reads and number of genera in respect of wastewater samples. The rarefaction curves demonstrated a good depth of coverage for wastewaters, with leveling of the curves by approximately 2000 reads. The same curves show differences between the different air samplers; in particular, "SKC_PBS_tank" and "Vivas_PS_tank" are those that reach the greatest number of sampled genera. Of the two, the "SKC_PBS_tank" reaches the highest number of genera but does not reach the plateau phase, failing to be representative of the bacterial community of our study. On the other hand, the "Vivas_PS_tank", while reaching a lower absolute number of observed genera, shows an early plateau phase, suggesting that the BioSpot-VIVAS sampler is able to capture a more representative sample of the bacterial community. These results are confirmed by the relative abundance barplots (Figure 1).

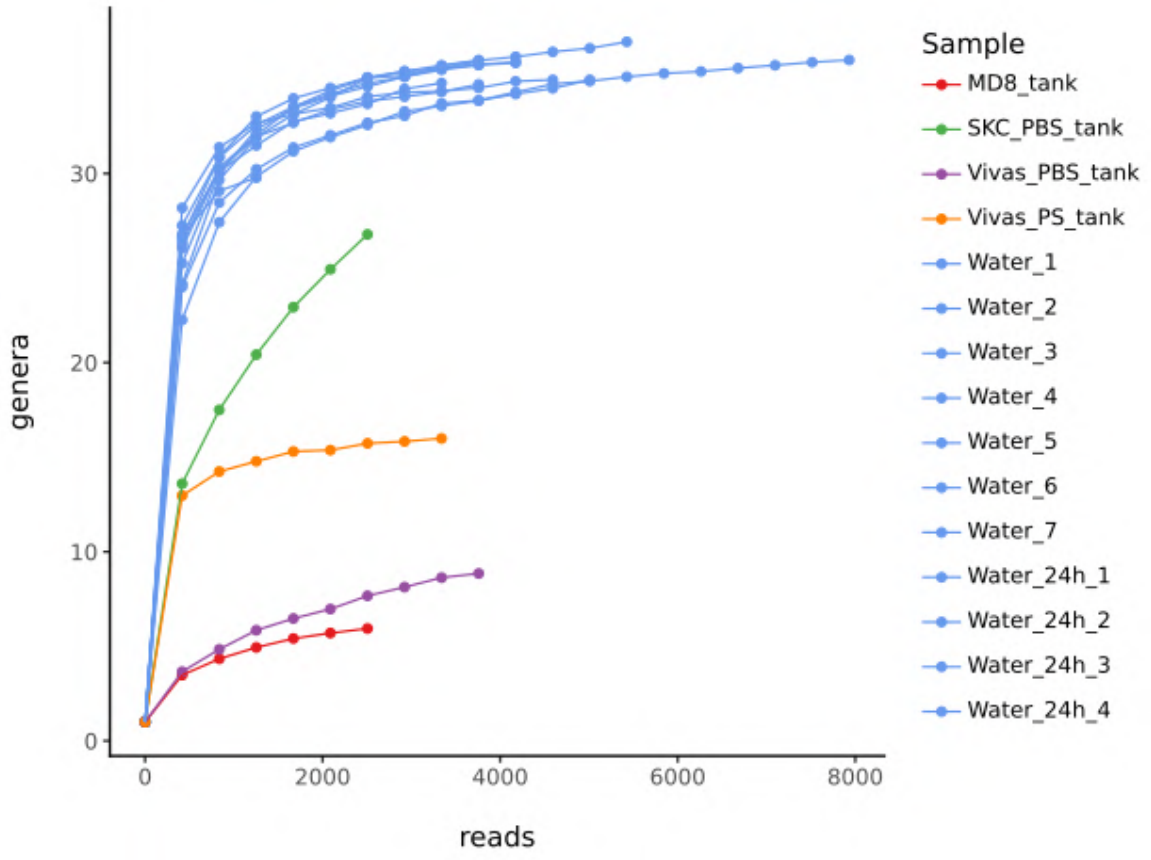

Figure 21. Alpha diversity rarefaction curves of bacterial communities based on the 16S rRNA gene sequences from air and wastewater sampled in the WWTP of Servola (Trieste). The x-axis represents the sequencing depth in number of reads, and the y-axis the estimation of the OTU richness detected at genus level. The air samples collected using the Airport MD8 has the sample ID "MD8_tank". The air sample collected using the BioSampler has the sample ID "SKC_PBS_tank". The air samples collected using the BioSpot-VIVAS have the sample IDs "Vivas_PBS_tank" (sampled using the PBS as collection media) and "Vivas_PS_tank" (sampled using the preserving solution of nucleic acids DNA/RNA shield™ as collection media). The wastewater samples that were collected by grab sampling for each day have the sample IDs "Water_1", "Water_2", "Water_3", "Water_4", "Water_5", "Water_6", and "Water_7". The wastewater samples that were the result of a composite process of sampling of 24 h in the same days have the sample IDs "Water_24h_1", "Water_24h_2", "Water_24h_3", and "Water_24h_4".

4 Discussion

With this study, we provide an analysis of the bacterial community present in the aerosol and wastewater of the aeration tank of the WWTP of Trieste, using the 16S rRNA metabarcoding technique, providing also new insights about different air sampling system devices.

4.1 Bioaerosol samples

Overall, the air samples show a lower number of genera than the wastewater ones in terms of relative abundance and alpha diversity rarefaction curves of the bacterial richness. Air samples are not as homogenous to each other as the water ones, and it seems that different sampling systems have different sampling efficiencies; therefore, the results obtained from a bioaerosol study may differ according to the air sampler used. In order to highlight and give support to the differences among the bioaerosol samples in the absence of replicates, we applied bootstrap and Euclidean pairwise distances to the community composition in terms of relative species abundances (Supplementary Materials Figure S1). Comparing air and wastewater samples of the same day, we found that the number of aerosolized genera of bacteria was lower than that present in the water of the aeration tank of the WWTP (62 vs. 78), but all the genera found in the air were derived presumably from wastewater. This can lead us to hypothesize that only a small part of the total number of bacteria is transferred from the liquid phase to the air, based on the ability of the microorganism to endure harsh physical stresses such as desiccation, UV exposure, or sampling stress. Many of the genera found in the air samples are composed of species that are capable of creating biofilms or that can even survive the treatments of a WWTP. These organisms can therefore, at the same time, be transported more easily from water to the air matrix and remain intact during the sampling phase. It is evident that microbial distribution patterns do not adhere to the widely accepted "everything is everywhere" theory, even though the data are still scarce. According to the findings, even if bacteria are microscopic, dispersal by air currents does not ensure that they reach every habitat equally, and arrival rates are low enough to be surpassed by local diversity due to natural selection. This can explain the fact that the air samples have a lower quantity of genera in respect to the grab wastewater samples.
We discuss below the six most abundant genera found in all the aerosol samples.
Stenotrophomonas is a genus of ubiquitarian bacteria, found mostly in soil, plants, and wastewaters [58,59]. It plays a significant ecological role in the nitrogen and sulfur cycles, and some species can provide benefits for plants, making them potential candidates for biotechnology uses in agriculture [60]. Nevertheless, a species called *S. maltophilia* is emerging as human pathogen causing fatal bacteremic infections and pneumonia [61]. It is known that *S. maltophilia* can endure in activated sludge of WWTPs, as well as chlorine or UV disinfection, because of its capacity to export toxic metabolites from the periplasm and to create biofilms, which serve as a protective barrier [62,63,64]. As a result, *S. maltophilia* can survive the WWTP processes; hence, it can be released into the aquatic environment, thus contributing to the spread of antibiotic-resistance determinants. *Klebsiella* is a genus of bacteria that can be found in a multitude of ecological niches, including soil, water, plants,

birds, and mammals, both free-living and host-associated. In humans, it is typically present in the nose, throat, skin, and intestinal tract and can cause urinary tract and bloodstream infections, sepsis, or pneumonia [65]. Recently, several strains of this genus have posed a serious threat to clinical and public health on a global scale, as *Klebsiella* gained the ability to acquire gene and mutations for antibiotic resistance or for increasing its virulence features [66,67,68]. These opportunistic pathogens can be released into the environment mainly through sewage, and it is documented that they can survive even after the treatments of the WWTPs [69]. For instance, *Klebsiella pneumoniae* can survive or even develop virulence and antibiotic resistance in sewage environment; in fact, identical *K. pneumoniae* sequence types have been reported in both clinical settings and wastewater [70,71]. The genus *Enterobacter* belongs to a variety of environmental habitats, from soil to water and sewage, but its species can be also natural commensals of the animal and human gut microbiota [72]. *Enterobacter* species are part of the ESKAPE group of bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which are directly referred to resistant nosocomial infections [73,74]. Due to their adaptation to the hospital environment and their ease of acquiring several genetic mobile elements, including resistance and virulence genes, these bacteria are usually linked to multidrug resistance [75,76]. Different species of *Enterobacter* genus have been found in air samples from the WWTP environment, like potentially pathogenic *Escherichia coli* [77]. The *Acinetobacter* genus is ubiquitous in nature, being found in soil, water, or animals and humans [78]. It can be frequently found in WWTPs due to their capacity to create biofilms on both biotic and abiotic surfaces, being therefore resistant to different environmental factors, such as desiccation and disinfectants [79,80]. Due to its resistance to environmental factors, it can be found also in the air matrix [81] and was found in the WWTP bioaerosol by Zhang and colleagues [81]. It is supposed that, in activated sludge, *Acinetobacter* spp. experience a change in metabolism, which may be a strategy used to survive and persist to the wastewater treatment process [82]. Many *Acinetobacter* species include opportunistic pathogens and are among the most frequent causes of hospital infections, and, in particular, they are now gaining interest because of their ability to acquire antibiotic-resistance genes [83,84,85,86]. *Delftia* strains have been isolated from different environments, including contaminated soil [87], wastewaters [88], WWTP bioaerosols [89], and hospitals [90]. The members of this genus are plant growth-promoting bacteria [91] and can transform or degrade several organic pollutants [92,93]. There is evidence on the possibility that metal contamination of natural environments may play a significant role in the spread of microbial antibiotic resistance; in fact, it is known that some *Delftia sp*. isolates may be resistant to a variety of antibiotics [94]. Members of the *Pseudomonas* genus are considered to be one of the most varied and widespread

groups of bacteria, found in a variety of natural, clinical, and artificial environments [95], as well as in WWTPs [96]. A key characteristic of *Pseudomonas* species is the ability to produce extracellular products like polymeric substances, which have been implicated in attachment processes, biofilm formation, and virulence [97]. Members of the *Pseudomonas* genus are thought to be able to acquire nearly all known antimicrobial-resistance mechanisms thanks to their genome plasticity [98]. Some are able to adopt resistant forms, like spores, to survive in hostile environments; thus, dormant organisms can be released into the environment, along with any resistance genes they may have acquired, for example, in hospital settings [99].

4.2 Wastewater samples

The results are consistent with previous wastewater studies, which found the genera *Acinetobacter* and *Arcobacter* as dominant and common members of WWTPs worldwide [100-105]. The *Acinetobacter* genus includes Gram-negative aerobic species generally found in soil, water, sewage, and also clinical environments. In fact, the genus increased interest in the health-care field for the emergence of multiresistant strains, some of which are pan-resistant to antibiotics [106]. *Acinetobacter baumannii* is one of the most prevalent causes of nosocomial infections [107], and it is considered the World Health Organization's number one critical priority pathogen for which new therapeutics are urgently required [108]. The genus *Arcobacter* has become increasingly important in medical, veterinary, and food safety fields because of its emergent enteropathogenesis and potential zoonotic agents [109], as well as for the increased prevalence of antibiotic-resistance strains [110]. In fact, some *Arcobacter* species may cause various diseases in animals, such as reproductive problems, mastitis, and gastric ulcers; and in humans, including gastroenteritis, bacteremia, peritonitis, and endocarditis [111]. The same description can be ascribed to the genus *Pseudarcobacter*, later synonym of *Arcobacter* [112]. The family of *Arcobacteraceae* can be found in a variety of habitats, mainly in aquatic environments like groundwater and sewage, but also in food and food-processing facilities [111,113-115]. It hosts both animals and humans, being some species linked with intestinal diseases, bacteremia, and peritonitis [116]. *Bacteroides* are the most predominant anaerobe bacteria in mammals gut flora, playing a main commensal role in the microbial food webs by processing complex molecules [117]. Although this genus provides valuable insights into bacteria in maintaining health, some species are also linked to significant human diseases being opportunistic pathogens in other body locations [118,119]. *Aeromonas* is one of the most common genera found in wastewater microbial community [120], and it can also colonize soil, freshwater systems, or aquatic animals like fishes [121,122]. The presence of this genus in surface waters is

linked to a high concentration of total and assimilable organic carbon, as certain *Aeromonas* species are reliable indicators of water quality and pollution. It is well known that some members of this genus acquired antibiotic resistance genes, and several studies have shown that *Aeromonas* may have the ability to generate and spread antibiotic resistance in various types of water bodies [123].

4.3 Comparison between air sampling system devices

For what concerns bioaerosol collection in this field experience, the activation of the three samplers and operational control for allowing the sampling of 1440 L of air, MD8 with a light and compact body provided ease of collection of microorganisms on filters in the field, treating and extracting the gelatine support in laboratory. The BioSampler has a glass sampling body connected by tubes to an external suction pump, which operates optimally by generating turbulence and transferring bioaerosol from the air flow to collection liquid when the PBS volume is 20 mL; the volume should be checked and eventually refilled. The BioSpot-VIVAS is compact but comparatively heavy, and the positioning of the Petri dish filled with collection liquid and the relative cooling system resulted in not being comfortable for handling at the WWTP aeration tank.

According to data from this preliminary study, the liquid medium appears to be the most effective method for bioaerosol sampling, being the BioSpot-VIVAS and the BioSampler more efficient in respect of the MD8 Airport. In particular the DNA/RNA shield™ as the medium outperforms the PBS; in fact, it was not even necessary to proceed with the purification and concentration of the samples using the DNA Clean & Concentrator®-5 Kit after the DNA extraction. The DNA/RNA shield™ medium was only used for the BioSpot-VIVAS sampler since; in addition to the costs related to a higher liquid collection volume for the BioSampler (20 mL) vs. the BioSpot-VIVAS (2.5 mL), it is a solution that, when shaken, forms foam, and so it is incompatible with the swirling motion of the BioSampler device. These first results suggest that the BioSpot-VIVAS sampling system in combination with the DNA/RNA shield™ medium is the best method for bioaerosol sampling, as the BioSpot-VIVAS sample collected in the PBS medium showed poor biodiversity. The BioSpot-VIVAS is the sampler that operates at a lower flow rate than the others (8 LPM), and it is known that this characteristic allows for a physical collection efficiency above 95% for particles from 8 nm to 10 mm [54]. Thanks to a low water vapor condensation strategy, small particles are enlarged, and enlarged aerosols are collected more efficiently, enabling the resistance of desiccation and maintenance of viability during sampling. For what concerns the BioSampler, even if in a smaller number of reads, it succeeded to express a high biodiversity, slightly more than the BioSpot-VIVAS sample stored in DNA/RNA shield™ (see Figure 2). The low genetic abundance of the sample may

be due both to the chosen medium (i.e., PBS), which is not preservative of the nucleic acids as the DNA/RNA shield™ is, and to the fact that the sampling volume of this device is 20 mL, in which the collected material is highly diluted compared to the 2.5 mL volume of the BioSpot-VIVAS sampler. In terms of operational characteristics for use in the outdoor environment, the Airport MD8 was the easiest sampler to use and clean. In fact, its dimensions and weight are smaller than that of the BioSpot-VIVAS, it is less fragile in respect of the BioSampler which has delicate nozzles, and it is entirely made of glass. The transportation on field of the BioSpot-VIVAS device is not easy in comparison with the other two instruments due to its weight (22.5 kg) and its dimensions (760 mm × 485 mm × 370 mm). The Airport MD8 does not require auxiliary equipment and has a reasonable duration of battery power; on contrary, the BioSampler needs to be connected to a high-suction pump, and both the BioSpot-VIVAS and the BioSampler systems need to be connected to an electrical power source.

5 Conclusions

Currently, no standardized methods for bioaerosol sampling exist, so using the metabarcoding approach to aerosol samples, the main implication of this study is that the present findings represent a first field experimental step toward the understanding of a significant diversity between different air sampling systems (i.e., filtration, swirling aerosol collection, and condensation growth tube), even if the sample size is critical to estimate a robust statistical power. Future directions in this sense could involve increasing the number of sampling days and, consequently, the number of samples for each device, given the operational availability in the plant.

In this screening comparison on bioaerosol samplers, we identified a candidate device and collection medium for the bioaerosol sampling, suggesting that BioSpot-VIVAS sampler with DNA/RNA Shield™ as the collection liquid is the best choice, despite lacking an optimal ease of operation due to the dimension, weight, and need for parameter tuning. Our data also highlight a difference in bacterial biodiversity between wastewater and air samples, with the latter being different depending on the device used based on the parameters set in this study (an overall sampling volume of 1440 L fixed for each sampler). All the genera found both in the wastewater and in the bioaerosol are consistent with other studies reported in the literature. However, since most of the studies reported in the literature are based on traditional cultural methods, which may result in an underestimation of the

microbiological richness, it is difficult to make a true comparison with the abundances of the bioaerosol samples. Furthermore, the majority of the bioaerosol studies in the WWTP environment do not use the same sampling devices employed in the present work.

Focusing on possible human pathogens, the metabarcoding technique, combined with an automated sampling methodology, can be modified to validate and improve epidemiological models, as well as to organize early warning systems in biomonitoring approaches. To implement the study of the biodiversity present both in air and wastewater a metagenomic survey can be followed to identify a vast number of genomes and perform a functional characterization present in the samples. In addition, in-depth physiochemical and microbial characterization of bioaerosol will be considered in future studies in parallel with the sequencing approach. By taking into consideration smaller aerosol particles (i.e., <0.3 μm), effectively collected by BioSpot-VIVAS, the viral component of the bioaerosol could be investigated as well.

6 References

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SECTION 5

Antimicrobial resistance analysis in the wastewater treatment plant of Trieste (Italy): a multidisciplinary study

Paper in preparation

Abstract

Wastewater Treatment Plants (WWTPs) are essential for mitigating environmental contamination and public health risks by treating municipal and industrial sewage. This study evaluates the WWTP in Trieste, Italy, focusing on its efficiency in removing pollutants, microbial load, and antibiotic resistance genes (ARGs). Influent and effluent waters were sampled for three days and analyzed using chemical and molecular biology techniques such as metagenomics. Chemical analyses identified various antibiotic residues and emerging contaminants, including macrolides, tetracyclines, fluoroquinolones, and sulfonamides. The WWTP effectively reduced several antibiotics (e.g., sulfamethoxazole, trimethoprim, azithromycin), though persistent compounds like fluconazole and ciprofloxacin showed minimal reduction. Notably, clindamycin concentrations increased in effluents, likely due to biotransformation during treatment. Biological analyses revealed significant shifts in microbial communities. Dominant genera in influent waters, such as *Acinetobacter* and *Acidovorax*, were largely replaced in effluents by resilient genera like *Mycobacterium* and *Zoogloea*. Effluent waters exhibited higher microbial diversity but reduced biomass, indicating selective pressures. Metagenomic analysis showed significant reductions in ARGs, including genes conferring resistance to tetracyclines (tet(Q)), beta-lactams (OXA beta-lactamase), and macrolides (msrE). However, residual ARGs in effluents highlight the need for enhanced treatment technologies. Co-occurrence network analyses revealed distinct ecological interactions and modular structures in microbial and ARG networks. Influent waters exhibited higher connectivity due to ARG-mediated interactions, while effluents showed more compartmentalized microbial communities, reflecting treatment-induced changes. This study highlights the role of WWTPs in reducing ARGs and contaminants while emphasizing the need for advanced treatment methods to address persistent pollutants. The findings demonstrate the utility of metagenomics in improving wastewater treatment strategies and mitigating antimicrobial resistance.

1 Introduction

1.1 The importance of wastewater treatment plants

Wastewaters are a mixture of liquid or waterborne waste originating from domestic, industrial, and commercial sources, combined with groundwater, surface water and stormwater runoff. Typically, it comprises a significant concentration of organic compounds and oxygen-demanding substances, inorganic chemicals, suspended solids, pathogenic microorganisms, and hazardous or toxic substances [1]. Wastewater treatment is a crucial process employed globally to manage municipal and industrial sewage. It refers to the procedure of removing contaminants from raw wastewaters prior to its discharge into aquifers or natural water bodies, such as rivers, lakes, and oceans. Since pure water does not exist in natural environments (unlike, for instance, in controlled conditions such as chemical laboratories), the classification of water as clean or polluted depends on the type and concentration of impurities, as well as the intended purpose. Generally speaking, water is deemed polluted when the concentration of contaminants is sufficient to make it unsuitable for specific uses. In order to reduce the surface water contamination from municipal wastewaters, the European Community started to state an environmental legislation (Council Directive 91/271/EEC 1991 as amended by the Commission Directive 98/15/EEC of 27 February 1998) [2], which means that the member states of the European Union must guarantee the monitoring of urban wastewater discharge and its impacts [3]. In order to reduce sanitary concerns associated to discharged effluent waters, the current Italian regulations (D.Lgs. 152/2006 of 3 April 2006) set wastewater treatment plant (WWTP) effluent emission thresholds for a variety of chemical compounds, overall toxicity and bacterial loads (mainly *Escherichia coli* concentrations) [4,5]. More specifically, the following limits are set by the Italian law: the content of active chlorine must be less than 0.2 mg/L, there must be no acute toxicity, and the concentration of *E. coli* must not exceed 5000 CFU/100 mL (recommended value). Since the concentrations of *E. coli* in the effluent can occasionally exceed the limit set by the authorities, many WWTPs use a wastewater disinfection process to adhere to this value. Since different effluents have unique chemical-physical properties and the reuse of the wastewater (i.e., for agricultural purposes) is sometimes used, the disinfection strategies in a WWTP should be examined case by case [6].
Recently, in November 2024, the Council of the European Union adopted a revised Urban Wastewater Treatment Directive aimed at enhancing the efficiency and effectiveness of wastewater treatment across member states. In particular, is now required the systematic monitoring of microplastics, antimicrobial resistance, and certain viruses in order to protect public health by enabling early detection of potential health treats [7,8].

Intuitively, effluents from WWTPs may not be entirely devoid of (human) pathogens, and the microbial community present in the effluent, as well as its associated nutrients, may actually facilitate the growth and spread of pathogenic bacteria within the environment. For instance, Wakelin et al. [9] demonstrated that the continuous effluent discharge, coupled with high nutrient concentrations in the sediment downstream from an Australian WWTP, significantly impacted the bacterial community composition in the sediment, leading to an increase in overall diversity. Furthermore, a research conducted in rural Bangladesh showed that fecal bacteria belonging to the genera *Shigella* and *Vibrio* were present in groundwater extracted from shallow tube wells [10], highlighting the possibility of fecal contamination of the natural environment resulting from anthropogenic effluents.

Sanitation of the influent wastewaters is crucial for preventing the spread of infectious diseases, as it concentrates the enteric microbial load of a population into a single waste stream for treatment. Thus, wastewaters become a rich source for disease monitoring and surveillance, reflecting the overall health burden of a population. As it emerges from review published in Trend in Analytica Chemistry [11], wastewater-based epidemiology (WBE) is an emerging field that is gaining considerable global interest, fueled by rapid advancements in analytical approaches based on high-throughput sequencing, bioinformatics, and biostatistics. With the use of these technologies, researchers may develop increasingly sophisticated methodologies for analyzing wastewaters, offering a comprehensive view of clinical health at community level.

Despite the potential of WBE, there are still significant challenges to overcome due to the complex and dynamic nature of microbial communities in wastewaters, and substantial lack of methodological standardization. Factors such as interspecies competition, predation, and environmental selection pressures can hinder the detection and estimation of specific pathogens. Additionally, some dominant taxa found in WWTP influents might have environmental origins, suggesting that they may not be a reliable indicator of human community health. Given the complexity of this environment, further research is required to determine the variables influencing microbial community dynamics in wastewaters, particularly in relation to seasonal variations and weather-related fluctuations [12].

While biological methods for wastewater treatment have been utilized for over a century, research into the microbiological aspect of these processes faced significant methodological challenges until about thirty years ago [13]. At that time, microbial genetic-based techniques were emerging as one of the most important tools in wastewater microbiology (such as those by [13–20]), and enabled scientists to analyze the composition and dynamics of microbial communities in these particular environments to identify the key microbial players involved in different treatment processes. In fact, historically, pathogen monitoring in wastewaters has mostly focused on effluents due to practical constraints and traditional methodologies. Despite the wide presence of microorganisms, assessment

of water quality often relied on a limited range of microbiological indicators, such as fecal coliforms and *E. coli* (total coliforms/fecal coliforms).

Researchers are still investigating microbial WWTP communities given their high complexity and yet unknown dynamics, that may be crucial for an improvement of WWTP operation at large scale [21]. A very relevant driver for further developments is represented by the revised EU directive on urban wastewater treatment, recently approved by the Council of Europe [8]. As a result, research has begun to expand this topic, but the scientific community typically operated as isolated projects, often lacking standardized methodologies and shedding light only on a very limited number of microbial species. In recent years, researchers have proven that microbial communities in wastewater systems carry a distinct human fecal signature, including various taxonomic groups that can provide insights into the health status of the community such as: Bifidobacteriaceae, Coriobacteriaceae, Bacteroidaceae, Lachnospiraceae and Ruminococcaceae [12].

Nowadays research focuses mainly on the study of a range of microorganisms of medical significance, including pathogenic bacteria such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter spp.*; and various bacteriophages [22–25]. Beyond bacterial pathogens, WBE has the potential to improve surveillance of enteric viruses that are commonly detected in municipal wastewaters such as *norovirus*, *enterovirus*, *adenovirus*, *astrovirus*, and *rotavirus* [26]. Past studies have demonstrated the advantage of monitoring wastewaters to gain a more comprehensive understanding of viral epidemiology at community level, enabling public health system to respond more effectively to outbreaks and potential risks [27–29]. In fact, infected individuals release substantial quantities of viral particles and continue to shed for up to 8 weeks, even after symptoms resolution. Eventually, such data can be used to design or assess control measures, including vaccines [29].

As already mentioned, wastewaters hold a wealth of biological and chemical information that reflects population health. Indeed, WBE can provide insights into human exposure to a variety of emerging contaminants (EC), including pharmaceuticals, pesticides, and industrial chemicals (see *Emerging contaminants in WWTPs* section). These substances collected from agriculture, industry, hospitals, and households to WWTPs may furthermore create favorable conditions for antibiotic resistance (AR). Evaluate AR and microbial health in wastewaters could represent an important frontier for public health research (see *Antibiotic resistance* section) [30]. Nevertheless, there is no regulatory standard for monitoring antibiotic concentrations and AR in effluent waters, where it is proven the presence of these elements even after the treatments of the WWTPs [31–34].

With advancements in metabolomics, WBE may evolve to include the measurement of biomarkers related to diet and pathologies, thus providing a more direct measure of community health. This

potential is consistent with the need for more holistic public health assessments that consider a range of environmental and lifestyle factors [11].

In summary, WBE represents a promising and versatile approach for improving environmental risks assessments, public health surveillance, and policymaking. WWTPs represent sampling sites with extraordinary potential for gaining integrated experimental data for community health studies.

1.2 Molecular biology techniques applied on WWTPs studies

Recent advancements in sequencing technologies, such as single molecule real-time sequencing, allow for improved characterization of bacterial communities in wastewaters. In fact, since recent years, culture dependent techniques have been used for the detection of microorganisms in wastewaters, even though these methodologies are limited to the viable and culturable species. This leads to low accuracy, and biased representation of the sample since most of the environmental bacteria are unculturable [35–38]. For these reasons, various alternative (non-standardized) analytical techniques are extensively employed to explore a range of microbial-related dynamics, which is essential for researchers to expand the knowledge of water pathogens and obtain valuable data. The most common advanced techniques in several research fields are flow-cytometry [39], fluorescence in situ hybridization (FISH) [40], and biosensors. Last but not least, the molecular biology-based techniques are emerging as important instruments for advancing the wastewaters community knowledge. In fact, these are proven to be an effective tool for the detection, the identification and quantification of microorganisms in water enabling rapid, sensitive, and precise analysis [41].

The 16S metabarcoding approach was initially employed to provide insights into bacterial composition, taxonomically describing the bacterial species present within a sample [42]. However, this approach is not free from drawbacks since it lacks the capacity to deliver detailed functional information as it does not provide any qualitative nor quantitative data on metabolites. In addition, the 16S metabarcoding technique is susceptible to PCR amplification biases, which can result in high false-negative detection rates [43]. This leads to not accurate and reliable abundance data. Precise species identification depends on the availability of published genetic sequences and the distinctiveness of the barcode used [44]. Furthermore, it is clear that the output data are limited only on the bacterial community, excluding other organism (i.e., viruses, fungi, plants, etc.), and the same 16S rRNA barcoding technique can be performed considering different regions of the genes. For example, to study the most dominant bacterial genera found in influent wastewaters, Ye et al. [45] analyzed only the V4 region of the 16S rRNA gene, meanwhile VandeWalle et al. analyzed the V6 region of the same gene [46]. On the other hand, full-length 16S rRNA gene sequencing can enhance

taxonomic identification at both the genus and species levels. On the other hand, shotgun metagenomics is considered the preferred technique to obtain more complete in-depth data on microbial taxonomy and associated functional roles. It represents an alternative approach that involves the direct random sequencing of the whole genomic DNA, rather than a small number of genetic markers, relying on Next-generation sequencing (NGS) technology [47,48]. It is considered as an unbiased approach since it does not require PCR enrichment of samples, therefore it allows to describe the complexity of the sampled environment [49,50]. However, environmental metagenomes are extremely difficult to assemble due to the huge amount of data of genomes representing thousands of species at unknown abundance distributions, many of which are closely related or are not represented in databases [51,52]. This latter approach could help the microbial profiles comparison of inflow and effluent of WWTPs, providing information on the dynamics of potentially pathogenic microorganisms.

1.3 Microbial community in WWTPs

The microbial community composition and diversity are influenced by the specific WWTP and the influent characteristics [53,54]. Additional factors, such as the capacity of the WWTP, and environmental conditions like temperature and rainfall, also play significant roles [54–56]. Effective treatment of municipal wastewater is a basic need to control infectious diseases and other environmental and health hazards. Although most pathogens and antibiotic resistance genes (ARGs) appear to be eliminated during WWTP processes, the presence of opportunistic pathogens in the effluent remains a concern. Studies indicate that effluents may still harbor human-derived bacteria and ARGs, posing risks to both human and environmental health [57,58]. For instance, in a water-scarce country like Saudi Arabia, where achieving 100% reuse of treated wastewater by 2025 is planned, it is crucial to perform microbiological studies not only in influent but also in effluent waters of WWTPs [59]. The assessments of potential risks related to water reuse should preferably be conducted using a metagenomic approach rather than relying solely on conventional microbiological quantification methods, such as coliform counts [60]. In addition, little information is available on their environmental fates in receiving water bodies [61].

Still limited studies have exanimated the microbial communities by comparing the influent and effluent of WWTPs with most research focusing on a few countries, including the USA, Hong Kong (China), and Spain [58,62–65]. Additionally, metagenomic characterization of wastewater studies is scarce, and most of them show relatively low taxonomic resolution, limiting the ability to identify

diverse microbial communities accurately. This constraint arises because molecular identification often focuses on short hypervariable regions of the 16S rRNA gene, which reduces phylogenetic resolution and hinders the identification of specific human pathogenic bacteria. Integrating complementary approaches could address these limitations and enhance the scope of these studies. [58,62–65]. More broadly, the bacterial community in WWTPs is predominantly composed of genera within the Proteobacteria phylum, such as *Arcobacter*, *Aeromonas*, *Corynebacterium*, *Clostridium*, *Dokdonella* and *Zooglea* [53,66]. A global study of 14 urban WWTPs across United States, Canada, China, and Singapore identified the bacterial genera *Arcobacter*, *Aeromonas*, *Corynebacterium*, and *Clostridium* in the majority of activated sludge, influent, and effluent samples using the 16S amplicon approach [45].

Studies conducted on different regions of the 16S rRNA gene show as most abundant in influent wastewaters the following pathogenic species: *Acinetobacter* spp., *Clostridium perfringens*, *Escherichia coli*, *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Staphylococcus aeureus*, and *Vibrio cholerae* [45,46,61]. Additionally, genera such as *Bacteroides*, *Acinetobacter*, *Macellibacteroides*, *Pseudomonas*, *Aeromonas*, and *Trichoccus* are frequently observed [63]. Further insights were provided by studies focused on specific WWTPs. Tong et al. identified *Arcobacter* as the most dominant bacterial genus commonly found in the influents of six WWTPs, with *Bacteroides*, *Acinetobacter*, *Macellibacteroides*, *Pseudomonas*, *Aeromonas*, and *Trichococcus* also prominent. The same study reported a decrease in abundance of *Pseudomonas*, *Aeromonas*, and *Acinetobacter* in effluents after the treatment [63]. Similarly, a metagenomic study in a municipal WWTP in Singapore containing membrane bioreactors, revealed that *Pseudomonas*, *Bacteroides*, *Aeromonas*, *Prevotella*, and *Cloacibacterium* genera were dominant in influent samples [58].

The viral component of the WWTP community also presents a substantial challenge, with viral particles concentrations ranging from 10^8 mL^{-1} to 10^{10} mL^{-1} [67,68], 10-1000 times higher than in other aquatic environments [69]. These viruses predominantly include enteric pathogens like Hepatitis E [70], Rotavirus [71], Rotavirus-A [72], Norovirus GI [73], Norovirus GII [74], Hepatitis A [74], Enterovirus [75], Reovirus [76], Adenovirus [73], and Astrovirus [77]. Most of them may also persist throughout the treatment processes of the WWTP and therefore be discharged into the environment [67,68].

In Italy, studies on microbial and ARG profiles of wastewaters are limited, and metagenomic characterizations are lacking. Research analyzing the 16S rRNA amplicon, such as a study monitoring

three WWTPs in central Italy [78], revealed that the predominant phyla in influent waters included Proteobacteria, Bacteroidota, Firmicutes, and Campylobacterota. Approximately half of the microbial community, in terms of relative abundance, consisted of only three genera: *Arcobacter* (22 %), *Acinetobacter* (15.5 %) and *Flavobacterium* (10.9 %). In the effluents, the same genera were dominating but with lower percentages. Another study conducted by Becsei and collegues [79] in Bologna and Rome (Italy) reported occasional blooms of *Pseudomonas*-dominated communities in influent waters, accounting for up to ~95% of sample DNA.

1.4 Emerging contaminants in WWTPs

Emerging contaminants (EC) represent a class of substances that have only recently been identified as proven or potential cause of adverse effects on human health and aquatic ecosystems. Carcinogenic, toxic, reproductive, cardiovascular, and nervous system disorders are among the possible harmful effects of ECs exposure [80]. These contaminants include a wide range of industrially synthesized substances, which are commonly found in various products, including pharmaceutical preparations, hormones, artificial sweeteners, antidepressants, nonsteroidal anti-inflammatory drugs, personal care products, and antibiotics [81]. Some of these substances, particularly pharmaceutical products, exhibit pseudo-persistence, which means that even if they are not really persistent in the environment because of degradation, they would eventually and effectively behave as persistent compounds due to their constant use and release into the environment [82]. Consequently, these substances have been detected in terrestrial freshwaters, drinking water, and wastewaters, and are subject to ongoing monitoring [80].

It is commonly known in pharmacology that organisms that consume antibiotics do not completely absorb their active molecule. Studies have shown that only 10–50% of the active compound of antibiotics is metabolized by humans and animals, while the remaining 50–90% is excreted as waste. These compounds reach the WWTPs via the sewer network, making the WWTP a critical site for the study of ECs [81,83–91]. Antibiotics that enter WWTPs can be removed primarily through chemical, physical, or biological degradation processes. However, the removal process of these compounds is not always complete, meaning that they may persist in the treated effluent discharged from the plant [92]. This depends on the chemical-physical properties of the substance but also on the technologies employed by the specific WWTP [93,94]. It is reported that the most common antibiotics found in WWTPs belong to the classes of macrolides, sulfonamides, trimethoprim and quinolones, followed by the groups of tetracyclines, β-lactam and lincomycins. More specifically, clarithromycin, erythromycin/erythromycin- H2O, azithromycin, roxithromycin, sulfamethoxazole, trimethoprim,

ofloxacin, ciprofloxacin, norfloxacin and tetracycline are the most detectable in WWTPs, due to their frequent prescription and intrinsic stable chemical structure [90,95].

As noted by Tran et al. [91], the majority of WWTPs for municipal water purification are primarily designed for removing or at least reducing to threshold levels concentrations of organic substances (i.e., nitrogen and phosphorus compounds). These plants are not specifically engineered to address EC, which have only recently been recognized as significant pollutants [90]. In WWTPs, only part of hydrophobic ECs is typically removed in the primary treatments, which are aimed at eliminating solid matter through progressively finer screening systems, as hydrophobic ECs tend to adhere to sludge. Additionally, ECs may be removed to a limited extent through volatilization, a process governed by Henry's Law which measures the volatility of a chemical [96]. It depends on the chemical and physical properties of the substance as well as the operational parameters of the treatment process, such as temperature and aeration. However, volatilization is generally considered a negligible removal mechanism for ECs in WWTPs [91].

Another concern about the presence of ECs in WWTPs, such as antibiotics and antimicrobial agents, is the development of AR (i.e. ARGs and AR bacteria), which reduces the therapeutic potential of antibiotics against human and animal pathogens [80,93,94,97]. AR is a selective process that occurs under conditions of constant exposure to antibiotic substances present in subtherapeutic concentrations, below the threshold defined by the minimum inhibitory concentration (MIC), which is a bacterium-specific parameter [97]. As previously said, these conditions are commonly found in WWTPs and are ideal for the development of ARGs [85] (see *Antibiotic resistance* section).

Bacteria can survive exposure to antibiotics by acquiring ARGs in any environment that supports their growth. Therefore, wastewaters can serve as a reservoir for the development of ARGs, promoting the survival and selection of resistant bacteria [92]. Usually a range of antibiotics can contribute to the creation of the same ARG, while a single antibiotic can trigger the development of multiple ARGs [81,98]. Within this context, WHO defined *ecopharmacovigilance* as an emerging science that encompasses the activities of detection, evaluation, understanding, and prevention of adverse effects related to the presence of pharmaceutical products in the environment [99]. It is thus a field focused on studying the long-term impacts of pharmaceuticals on the health of ecosystems. Ecopharmacovigilance provides also for more sustainable drug production and management practices [100,101]. Drugs, before being commercialized, must pass the Environmental Risk Assessment (ERA), a preliminary evaluation to assesses their potential impact on the environment [102]. However, it was only in the late 1990s that studies began to investigate the concentrations of pharmaceutical substances entering and exiting WWTPs. Despite the adoption of the European POSEIDON project in 2005, which aimed to standardize the analysis of pharmaceutical

concentrations in WWTPs, no desired result was achieved. The data collected were limited to just 13 substances and were not comparable across countries due to differences in the number of plants considered and the duration of monitoring [103].

1.5 Antibiotic resistance

The field of WBE continues to evolve and is expected to play a significant role in addressing pressing public health challenges [93,104–109]. Given the ongoing hazards posed by AR bacteria and pathogens that may be in effluents, the surveillance of treated water of the WWTPs is crucial [110,111]. Antibiotics are widely used to prevent and treat infections in humans, animals, and plants, but their high and incorrect consumption have made them increasingly ineffective due to antimicrobial-resistant microorganisms emerging and spreading globally [112–114]. AR was announced by the World Health Organization (WHO) as one of the top global public health threats facing humanity, since it leads to prolonged hospital stays, higher medical costs, and increased mortality.

AR is defined as the ability of a bacterium to resist an antimicrobial agent following standard treatment [115]. It was first detected in the late 1950s to early 1960s among enteric bacteria, i.e., *E. coli*, *Shigella* and *Salmonella* [116,117]. It is a phenomenon that has existed since before the introduction of antibiotic drugs for human health use and the ARGs possessed by human pathogenic bacteria are far fewer than the total ARGs found in nature [97]. Under environmental conditions, bacteria naturally develop genes capable of conferring AR capabilities through evolutionary processes. However, the abuse of antibiotics for medical purposes, the carelessness in adhering to prescribed antibiotic therapies, and the widespread use of these substances in livestock and agricultural fields further led to enhancing this kind of mutations in bacteria [83,84,88,91,118]. Some multi drug resistant (MDR) species globally important include hospital-acquired pathogens like *Mycobacterium tuberculosis*, *Enterococcus faecium*, *E. faecalis*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [119–121]. These bacteria have the ability to propagate new genes that confer antibiotic-resistant properties through generations (vertical gene transfer, VGT) and horizontal transfer with other bacteria in ecosystems [89]. Horizontal gene transfer (HGT) can confer pathogenicity to completely harmless bacteria [86] and occurs through the transfer and acquisition of genetic material such as DNA strands, transposons, plasmids, integrons, and bacteriophages in the processes of transformation, transduction by bacteriophages, and conjugation between two bacteria [83,88,118,122]. Direct contact between

bacteria is not necessary for HGT to occur; the genetic material can be free in the environment or in the biofilm created by the same bacteria. The HGT can occur also between bacteria belonging to different taxonomic or ecological strains, leading to the development of multi-resistances that can spread into the environment [97]. The mechanisms of ARGs often work against a particular class of antibiotics. It is estimated that at least 70% of pathogenic microorganisms exhibit resistance to at least one antibiotic, particularly to broad-spectrum antibiotics commonly used as first-line treatments [115,123]. AR can act according to three different main mechanisms: the first acts by limiting the concentrations of antibiotic substances inside the cell, which either prevents the antibiotic from entering the cell or promotes its expulsion through efflux pumps before it reaches its target inside the pathogenic cell (e.g. a lack of the imipenem transporter OprD2 provides resistance to this antibiotic in *Pseudomonas aeruginosa*). The second mechanism involves the degradation or deactivation of the antibiotic by producing beta-lactamase, an enzyme that hydrolyzes the beta-lactam ring present in the structure of some antibiotics, like penicillins. The third and last mechanism consists in the production of an alternative target to which the antibiotic is directed, thus blocking its function by target mutation, replacement, protection or enxymatic modification [92,97,115,123].

According to the European Centre for Disease Prevention and Control (ECDC), it is estimated that in Italy approximately 11,000 deaths per year were caused by AR between 2016 and 2020, while at the European level, the number rises to about 36,000. These data exceed expectations and are currently comparable to the combined deaths from influenza, tuberculosis, and HIV/AIDS [124]. On a global scale, the WHO [125] estimates that in 2019, AR was directly responsible for 1.27 million deaths, and contributed to a total of 4.95 million deaths. This global emergency affects both high-income and low-income populations and will lead to a one trillion dollar increase in global healthcare costs by 2050 due to the need for more intensive and expensive treatments. The Istituto Superiore di Sanità (ISS) forecasts that AR will cause 10 million deaths annually by 2050 [126]. As evidenced by the first documented detection of water contamination by AR agents in the 1970s in the United States, it is evident that AR is a critical issue that has only recently gained global attention. The global interest on AR gained further momentum with the Global Antimicrobial Resistance and Use Surveillance System (GLASS) launched by the WHO in 2015. This first global collaborative effort was thought to standardize the monitoring of AR, facilitating more robust scientific research [127].

One of the factors that contributes to the spread of AR is the fact that high amounts of antibiotics and antibiotic resistance bacteria get into sewage and, consequently, into WWTPs, where their removal efficiency is still insufficient. Moreover, the presence of antibiotics in sub-inhibitory concentrations creates conditions for selective pressure between bacteria, which might become resistant strains.

Therefore, it is believed that the WWTPs are reservoirs (so called “hotspots”) of ARGs [107,128,129]. The AR microorganisms, along with ARGs, can be released from the WWTP into aquatic environments such lakes, rivers, and oceans, contributing significantly to their subsequent spread among humans, animals and plants [130–133]. The mechanisms that may play a role on the ARGs content within a WWTP are detailed in Figure 22. It is known that the treatment process leads to changes in the final *resistome* (which refers to the total set of ARGs present within the microbial community [134–137]) of the effluent: wastewater purification reduces or removes chemical contaminants, which serve as environmental pressures on bacterial populations. This can result in shifts in the bacterial community across the plant’s various stages, with alterations in bacterial dominance observed from influent to effluent treatment [85]. Furthermore, disinfection operations can lower the bacterial load, potentially reducing biomass while simultaneously increasing the relative abundance of ARGs in the treated water, due to the reduced concentration of antibiotics compared to the influent water entering the treatment facility. Additionally, the frequency of HGT between bacteria is significantly higher in wastewater environments than in typical natural ecosystems, being WWTPs anthropogenic systems where urban sewage is processed [138]. As a result, bacteria will have a higher chance of interacting with numerous pathogens that may carry genes that confer AR properties [84,97].

The reduction of ARGs in the effluent waters (i.e., after the purification processes of WWTPs) in comparison to raw influent wastewaters is documented in various studies [81,139–141], while many others showed a comparable number of ARGs in the two districts of WWTPs. However, the enrichment of some ARGs in effluent in respect to influent waters is observed in some studies, probably due to the selective conditions of this kind of environment (as mentioned before in this chapter) leading to selective advantages for ARGs and HGT among bacterial community [141–143]. In a research conducted on final effluent wastewater samples in Germany, 123 clinically relevant ARGs were detected, including aminoglycoside, β-lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, tetracycline, trimethoprim, and sulphonamide resistance genes, as well as genes encoding multidrug efflux pumps capable of conferring resistance to wide variety of compounds [108].

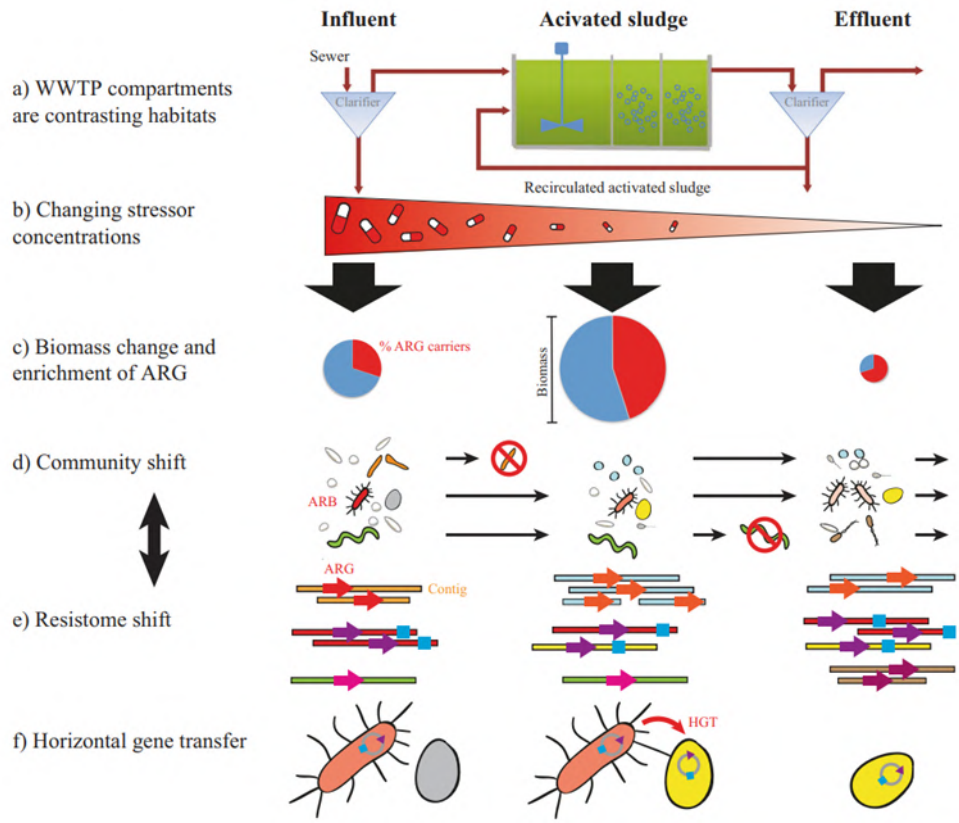

Figure 22. Key mechanisms affecting the resistome during the purification processes in a WWTP [85]

Various environmental bacteria, including *Acinetobacter* spp., *Aeromonas* spp., and *Pseudomonas* spp., are prone to develop multidrug resistance [144,145]. These human-associated bacteria have adapted to humid, diverse aquatic environments [146–150] and are common components of the microbial communities found in municipal WWTPs [151,152]. For example, Zhang et al. [144] have documented an increase in antibiotic-resistant *Acinetobacter* spp. within WWTPs. Other studies conducted in Hong Kong have identified the presence of pathogens such as *Clostridium perfringens* and *Mycobacterium tuberculosis*-like species in WWTP effluents, highlighting the importance of continuous monitoring [153].

The issue described above can be considered one of the largest emerging global concern due to its influence in multiple areas (i.e., both human and ecosystem health), aligning with the One Health concept [154] (Figure 23).

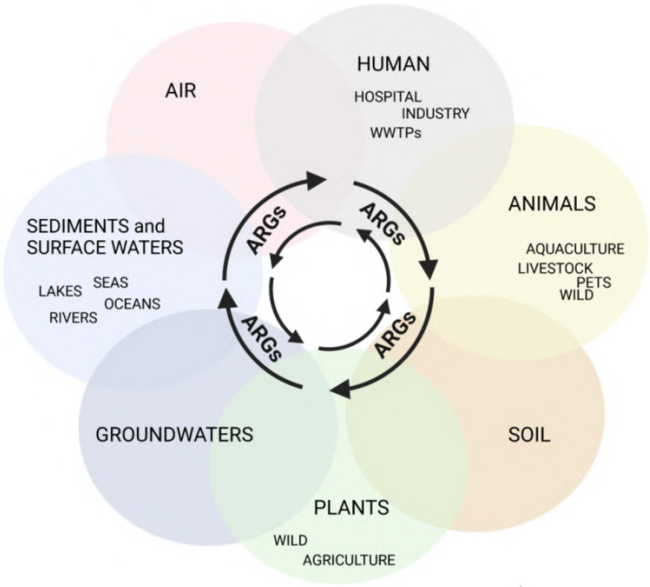

Figure 23. Routes of ARGs transmission between different environments [155].

Shotgun metagenomics data can offer valuable insights into the interactions and co-occurrence of ARGs within a controlled population or environment [137]. The resulting metagenome, i.e., the whole NGS reads obtained from a sample, can be analyzed to identify and characterize the resistome. The metagenomics network approach addressed to the problem of antibiotic resistance has been used in many researches on microbial species found in the gastrointestinal tract [156], soils [157], and WWTPs [158,159].

According to literature, the most commonly detected ARGs in WWTPs (in both influent and effluent waters) include the genes resistant to β- lactam (bla_{CTXM}, bla_{TEM}, bla_{OXA-A}, bla_{SHV}, mec_A), quinolone (qnrS, qnrC, qnrD), sulphonamide (sul1, sul2, dhfr1), tetracycline (tetA, tetB, tetE, tetG, tetH, tetS, tetT, tetX), macrolide (ereA, ermB, ermC, erm43) and class 1 integron (int1). Among them, bla_{CTXM}, bla_{TEM}, sul1, sul2, tetO, tetQ, tetW and ermB are the most frequently reported ARGs [88,106,139,141,142,160–162].

1.6 The WWTP of Trieste: case study

The WWTP of Servola is located in the Municipality of Trieste, in the Autonomous Region of Friuli-Venezia Giulia, within the industrial port area of the Servola district (*Figure 24*).

Figure 24. The WWTP of Servola (Trieste, Italy)

The plant is of mixed type as it collects both rainwater and wastewater, and it serves 199,305 inhabitants of Trieste [163], for a maximum of 5 million liters of wastewater treated per hour. Referred to as "the purifier that speaks to the sea", this plant is particularly distinctive due to its ability to modulate the intensity of wastewater purification in response to nutrient levels needs. This is aligned on the needs of the waters of the Gulf of Trieste, which are under the monitoring of the National Institute of Oceanography and Applied Geophysics (OGS).

The wastewaters are firstly subjected to a chemical-physical treatment, followed by the biological one (sludge section), where a heated anaerobic digestion is foreseen with the recovery of the biogas produced.

The first three treatments involve some pretreatment techniques, in which the influent wastewaters are subjected to physical treatment. The first consists in the elimination of solid bodies through grids (ISPRA, 2024), the second and the third include sand and oil removal by introducing air flows into the tanks allowing the oils to rise to the surface and the sands to fall to the bottom, to be then disposed of. New technologies like the Sedipac D have been introduced, which allows primary sedimentation using plastic material equipped with lamellae within which the water is treated [164]. Here, organic-nitrogenous particles of the order of a micrometer are deposited on the bottom.

The secondary treatments, the biological ones, play a crucial role in the wastewater purification process. These entail the removal of organic and nitrogenous compounds which, if highly concentrated, may adversely affect marine ecosystem upon the discharge of the treated water into the sea. The initial stage involves the Biofor® technology and requires a substantial supply of oxygen [165]. Here the bacterial colonies adhere to microspheres of Biostyrene facilitating the conversion of nitrogen compounds into sludge thanks to oxidation, nitrification, and denitrification processes.

Conversely, the second stage (post-denitrification) takes place in an anoxic environment, where bacterial colonies aggregate around small spheres of Biolite (Biostyr™ technology) to promote the biodegradation of residual organic and nitrogen compounds, which are likewise transformed into sludge [166]. Finally, after the biological treatments, the wastewaters undergo disinfection through exposure to ultraviolet (UV) rays, effectively eliminating any residual bacteria. The treated water can be then discharged to the sea via a duct that extends approximately 7 km into the Gulf of Trieste, where it is released homogeneously through a system of 600 "submarine release towers" (*Figure 25*).

Figure 25. Duct that extends approximately 7 km into the Gulf of Trieste, where releases homogeneously the treated water of the WWTP of Trieste through a system of 600 "towers".

As mentioned before, the wastewater treatment process also involves the production of sludge, composed of bioproducts from the treatment process in the tanks, which, after centrifugation, are used in the biogas plant for electricity production. Additionally, the solid waste resulting from this process undergoes further chemical treatments and is subsequently employed as soil fertilizer.

Aim of the study

The aim of the present study is to characterize influent and effluent waters of the WWTP in Trieste (Italy), focusing on antimicrobial molecules, microbial communities, and ARGs, by employing advanced chemical analyses, i.e., UHPLC-HR Orbitrap MS, and molecular biology techniques, i.e., metagenomics. This research aims also to elucidate the dynamics of microbial community composition and ARG profiles, providing insights into treatment efficacy and the environmental and public health implications of residual contaminants.

2 Materials and methods

2.1 Experimental design

Samples of influent and effluent waters were collected on May 8, 9, and 10, 2023, at the WWTP of Servola (Trieste, Italy). Each day, 1 L of influent water and 3 L of effluent water were sampled at the same time, resulting in a total of 6 samples (Figure 26, Figure 27). The bottles were maintained on ice until the arrival at the laboratory, where two different procedures were adopted for the preparation of the influent/effluent samples.

For the laboratory chemical analyses, 250 mL sub-samples of influent and effluent wastewaters were collected from the original samples and sent to ARPA FVG (Udine, Italy) for chemical analysis.

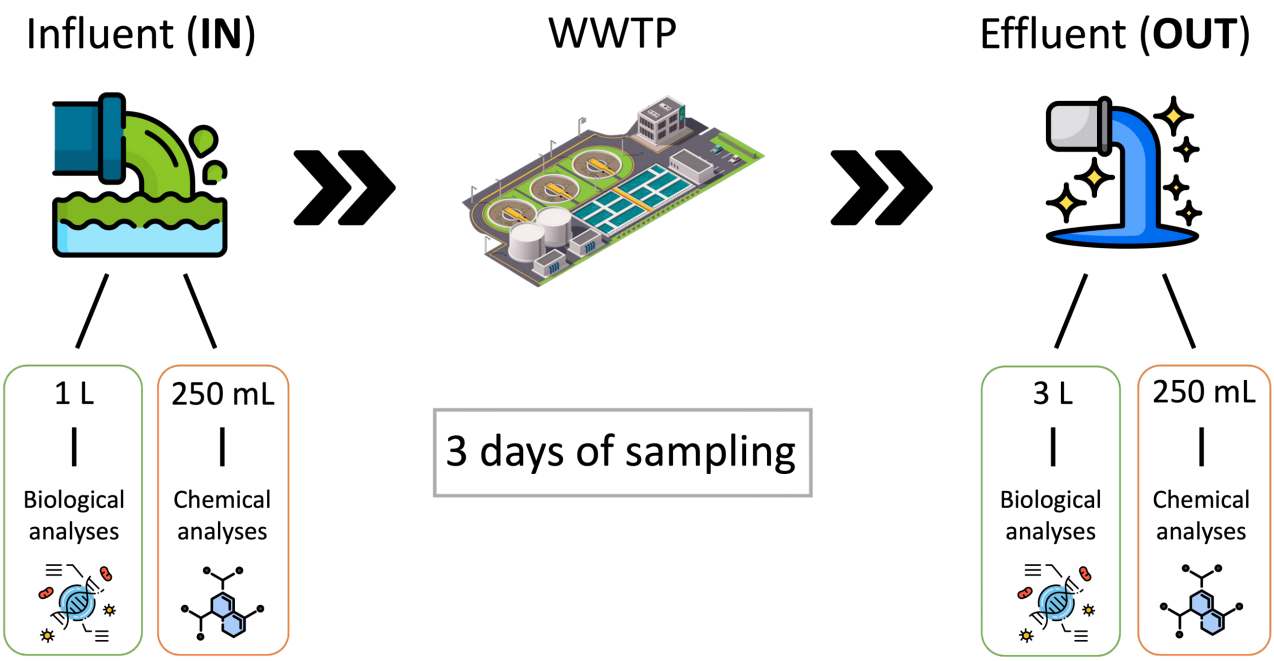

Figure 26. Experimental design of the study.

Figure 27. Sampling sites at the WWTP of Servola (Trieste, Italy). "IN" refers to the site where influent water was sampled, "OUT" refers to where effluent water was sampled

2.2 Chemical analysis

The samples underwent centrifugation at 13,000 rpm in Eppendorf tubes, and the resulting pellet was analyzed using UHPLC-HR Orbitrap MS for both targeted and untargeted analyses.

The targeted analyses were conducted by using the "TraceFinder 4.1" software, which enabled the analysis and quantification of antibiotic target compounds using standard solutions. The output consisted of concentration values (µg/L) for nine antibiotics.

The untargeted analyses, representing a novel and recently developed approach, utilized high-resolution mass spectrometry to examine all compounds present in the investigated samples (without relying on reference standard solutions). For this type of analyses, the "Compound Discoverer 3.3.2.31" software was employed. Its output provided values representing the areas under the peaks of concentration processed by the instrument. This approach is particularly suitable for investigating the chemical substances present in wastewater from a treatment system [167].

Upon completing the analyses, ARPA FVG reported the results to the Department of Chemical and Pharmaceutical Sciences at the University of Trieste. The concentrations were reported with different units of measure for different molecules, analyzed with either targeted or untargeted approaches. To better compare these quantitative data, a normalization of the values was performed by z-score. The use of z-scores standardizes the data by centering it around the mean and scaling it by the standard deviation, enabling a direct comparison of relative changes across compounds despite differences in their absolute concentration ranges. Visualizations of the data to allow a first inspection of the differences between influent and effluent concentrations of antimicrobials were built by using a boxplot and performing a Principal Component Analysis (PCA), which also allows to inspect the factors with major contributions to the differences between the samples. A statistical comparison of the changes in concentration between influent and effluent samples was performed by Mann-Whitney U test for each molecule and multiple test correction was applied by the Benjamini-Hochberg False Discovery Rate (FDR) method. Statistical significance was evaluated for FDR values <0.01.

2.3 Bioogical analysis

2.3.1 Pre-treatment of the samples

Each influent water sample (1 L) was divided in four bottles of 250 mL and was centrifuged at 8000 g for 15 minutes. Pellets were collected and transferred into a fresh tube and stored at -80 °C until further analyses.

Effluent waters, due to their lower concentration of particulate matter, were vacuum filtered on two 20 µm pore size cellulose filters per sample, for a total of 2 L. Filters were placed in sterile Petri dishes sealed with parafilm and stored at -80 °C until analyses.

2.3.2 DNA isolation, quality check, and sequencing

Technical replicates of all samples were performed in order to be finally pooled together and guarantee a sufficient DNA concentration for the metagenomic analysis.

For the influent samples, 6 technical replicates were prepared for each sample, and total DNA was extracted from each using the CTAB protocol, with an initial grinding treatment of the pellets in liquid nitrogen. For the effluent samples, a total of 8 technical replicates for each sample (the two filters per each day were cut in in four pieces) were processed for the extraction of the total DNA.

The CTAB protocol was performed for each sample as follow: the sample material was grinded to powder in presence of liquid nitrogen; then 500 μL of lysis buffer were added, and samples were incubated for 1 hour at 65 °C (mixing occasionally). After incubation samples were centrifuged for 5 min at 12.000 rpm, and the liquid phase was collected. Subsequently 500 μL of CI (Chloroform/Isoamylalchol 24:1) were added to supernatants under the hood, and the mixture was vortexed briefly and centrifuged for 5 min at 12.000 rpm (this step was optionally repeated). The upper phase (with DNA) was then carefully collected with a pipette and transferred into a new tube. Samples were then added with 1 mL of precipitation buffer, mixed gently, and left to rest for 1 hour at room temperature (RT). The samples were then centrifuged for 15 min at 12.000 rpm (RT). The supernatant was discarded, and the pellet resuspended in 350 μL of NaCl. Ten 500 μL of CI were added, and samples were vortexed and centrifuged for 5 min at 12.000 rpm. The upper phase was carefully transferred into a new tube; added with 210 μL of cold isopropanol and mixed carefully, and incubated overnight at -20 °C. The day after samples were centrifuged for 20 min at 4 °C and 12.000 rpm; the supernatant was discarded, and the pellet was dried in a vacuum centrifuge (for 10 min) or in an oven (for ca. 20 min) at 45 °C. Finally, the dried pellet was resuspended in 25 μL of $_{dd}H_2O$ or TE buffer.

Influent and effluent water samples were processed in separate hoods to avoid cross-contamination, with a blank DNA extraction performed as a control to ensure no environmental contamination. All samples were eluted in 10 mM TRIS. Finally, the replicates were pooled together to have one single sample for each day for both influent and effluent waters.

After the isolation, DNA quality was assessed by agarose gel electrophoresis and quantified using the NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

A treatment with RNase A was performed in order to get RNA-free samples, as follows: for each sample, 10 μL of RNase A were added, then samples were vortexed at maximum speed for 15 seconds and incubated at 60 °C for 10 minutes.

Lastly, DNA samples were purified through a size-selection strategy using magnetic beads following the Mag-Bind® TotalPure NGS kit (Omega Bio-Tek, Norcross, GA, USA) at a 0.4:1 bead ratio to

remove short length sequences. Samples were run on agarose gel electrophoresis and quantified with the Qubit™ Fluorometer (Thermo Fisher Scientific) using the High Sensitivity protocol.
All 6 samples (three influent samples and three effluent samples) were sent to the BMR Genomics srl (Padova, PD, Italy) for shotgun libraries preparation and high-throughput metagenomics sequencing on an Illumina NovaSeq 6000 platform with a 150 bp paired-end sequencing strategy and estimated output of 5 Gbases/sample.

2.3.3 Reads pre-processing

The raw reads sequenced by the facility were quality checked with FastQC v. 0.12.1 [168] paired with MultiQC v. 1.14 [169]. FastQC is a tool used to assess the quality of sequencing data by providing summary statistics and visualizations, while MultiQC aggregates reports from multiple samples into a single comprehensive report. According to the result of this step, the raw reads were trimmed using fastp v. 0.23.4 [170] with the following parameters: heading nucleotide trim (-f, -F) = 15 nt; trailing nucleotide trim (-t, -T) = 5 nt; remove homopolymers (-x); sliding window quality check on both strands (-5, -3); allowed "n" characters in read (-n) = 0; minimum mean quality in read (-e) = 20 nt.
These trimming parameters were chosen to ensure high-quality reads. The heading and trailing trims (-f, -F, -t, -T) remove biased bases at read ends. Homopolymer removal (-x) reduces errors from repetitive sequences. The sliding window check (-5, -3) maintains quality across the read, while setting allowed 'n' characters (-n) to 0 removes reads with ambiguous bases. Finally, the minimum mean quality (-e) of 20 balances read quality and data retention.

2.3.4 Metagenome-free analysis

Prior to metagenomic assembly, the trimmed reads were used in a "pseudo-metabarcoding" approach to obtain insights about the bacterial community composition in all the samples. Reads were mapped to the QMI-PTDB (June 2021) database provided within the microbial genomics module of CLC Genomic Workbench v. 23 [171]. This database pairs sequence data with taxonomy information for bacteria, allowing fine-grained taxonomy assignment and quantification. The resulting counts were used to compute ecological parameters of the influent and effluent communities at family, genus, and species aggregation level, i.e., total operational taxonomic units (OTUs), Shannon entropy, Pielou's evenness, and dominance [172].

Similarly, the reads were mapped to the Comprehensive Antibiotic Resistance Database (CARD) [134,173] to detect and quantify ARGs in our datasets. ARGs counts from each sample were then

grouped according to their source (influent and effluent), normalized with the upper quartile method, and the abundance of ARGs in the two groups were compared using a generalized linear model (GLM) approach. Statistical significance was assessed using an FDR-corrected p-value threshold of 0.05 and a fold change threshold of 2.

Association between bacteria and ARGs can be visualized by network analysis [174]. Using abundance data aggregated by genus (considering genera having a total maximum number of reads > 1000), co-occurrence networks were built separately for the two conditions (influent and effluent water samples) using the Spearman rho (ρ) coeffient. The co-occurrence matrices were filtered by $|\rho| > 0.6$ and p-value < 0.01 [175] to determine correlation degree. Networks were built by using genera and ARGs as nodes and adding an edge between two nodes when the filtering conditions were met. ARGs and microorganisms that reach a correlation coefficient threshold can be linked with lines to visualize correlation patterns and possible hosts for ARGs could be speculated [176] . Visualizations of the co-occurrence networks were created through the Fruchterman-Reingold layout in the Gephi software for easier interpretation [177]. For each network the modularity parameter was computed in order to compare them.

Co-occurrence networks were also built using correlations between taxa and ARGs abundances, therefore creating networks containing all taxa-ARGs, taxa-taxa and ARGs-ARGs relationships. The same filters described above were applied. Building this meta-network enabled the identification of key microbial lineages strongly associated with specific resistance genes, the detection of potential ARGs transfer hubs, and a more comprehensive understanding of how the treatment process affects both taxonomic and functional connectivity within the microbial community.

2.3.5 Metagenome-based analysis

The trimmed reads were assembled with different assembly methods to determine the most suitable for our use case. Specifically, two assemblers were tested: megaHit v. 1.2.9 [178] and metaSPAdes v. 3.15.5 [179] in the following modes:

- Individual assembly: each sample was assembled independently.
- Grouped co-assembly: samples from influent and effluent were assembled together, grouped according to their source.
- Co-assembly: all samples were assembled together.

Additionally, the meta-plasmid mode of metaSPAdes was exploited to try and assemble the plasmids in our samples in the same modes described for the main metagenome assembly.

The metagenome assemblies were compared using the following metrics: number of assembled sequences, Nx value, N50, and distribution of assembled sequence length. The number of assembled

sequences indicates the total contigs formed. The Nx value represents the sequence length such that x% of the total length is contained in contigs of at least this length. N50 is a common metric indicating the sequence length where 50% of the total genome length is in contigs of at least this size. The distribution of assembled sequence length helps assess the range and quality of the assembled contigs. Given the results of this comparison, the megaHit assembler performed better and successfully built the co-assembly with the available computational resources, whereas metaSPAdes failed due to limited computational resources. The total co-assembly produced by megaHit resulted as the best approach, allowing for effective direct comparisons of samples downstream. It will be referred to as "assembly" from now on.

The assembly was binned and analyzed with metaWRAP v. 1.3.2 [180] with the following modules:

- Binning, by combining MetaBAT [181], MaxBin [182], and CONCOCT [183].
- Bin refinement.
- Blobology (which creates GC vs abundance plots of contigs and bins) [184].
- Reassemble bins.
- Quantify, classify, and annotate bins.

This workflow provided a complete functional annotation of the metagenome-assembled genomes (MAGs).

Furthermore, the assembly was annotated for ARGs using RGI v. 6.0.3 with the CARD database [134,173]. Detected ARGs were finally quantified by using the bin abundances as a proxy. Comparisons of quantitative data were performed using a GLM-based differential abundance test, with significance determined based on an FDR-corrected p-value of 0.05.

This analysis is still ongoing, and the results presented in this chapter should be regarded as preliminary.

3 Results

3.1 Chemical analysis

The quantitative data provided by ARPA FVG were expressed as concentrations in μg/L for certain compunds (Azithromycin, Ciprofloxacin, Clarithromycin, Clindamycin, Fluconazole, Levofloxacin,

Sulfamethoxazole, Tetracycline, and Trimethoprim) or as peak areas processed by the UHPLC-HR Orbitrap MS (2'-Deoxyadenosine, Acycloguanosine, Ethambutol, Linezolid, Mycophenolic acid, N4-Acetylsulfamethoxazole, N-Desmethyl Clarithromycin, and Sulfapyridine).

To have a preliminary comparison between concentrations in influent and effluent samples, z-scored concentrations are reported in Figure 28. The WWTP effectively reduces the overall concentrations of several antibiotic drugs, such as acycloguanosine, sulfamethoxazole, sulfapyridine, N4-acetylsulfamethoxazole, clarithromycin, the metabolite N-desmethyl clarithromycin, mycophenolic acid, linezolid, tetracycline, trimethoprim, and azithromycin. Molecules such as fluconazole, ciprofloxacin, ethambutol, and levofloxacin exhibit minimal to no change in concentration after the treatment of the WWTP. On the other hand, Clindamycin and 2'-Deoxyadenosine display an apparent increasing concentration after the treatment.

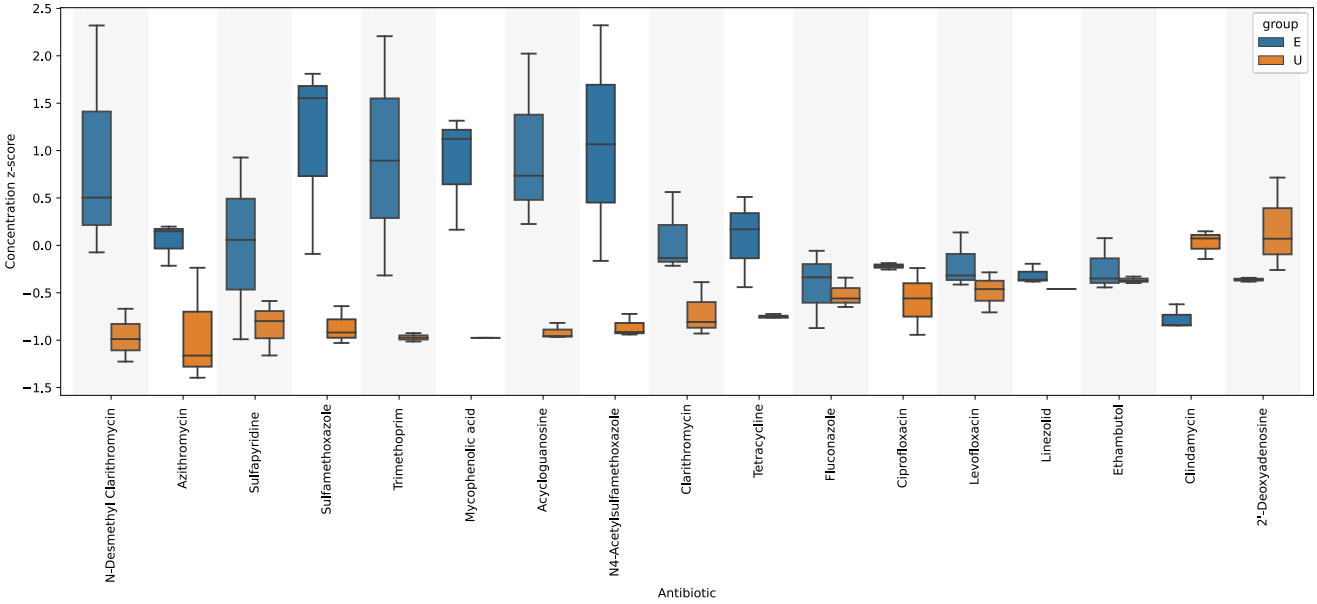

Figure 28. Boxplot of the z-scores of quantitative antibiotic data. Antibiotics are reported on the x axis, while the y axis reports the z-scores concentrations data. The blue color (E) represents influent data concentrations, while the orange color (U) represents effluent data concentrations.

The PCA plot (Figure 29) obtained from these data shows a clear clustering based on sampling source (group) on the first component (PC1), which explains 60.28% of the total variance. The second component (PC2, 18% of variance) shows clustering based on the day of sampling. The top five loadings are represented by the following antibiotics: mycophenolic acid, N4-acetylsulfamethoxazole, sulfamethoxazole, trimethoprim, and tetracycline.

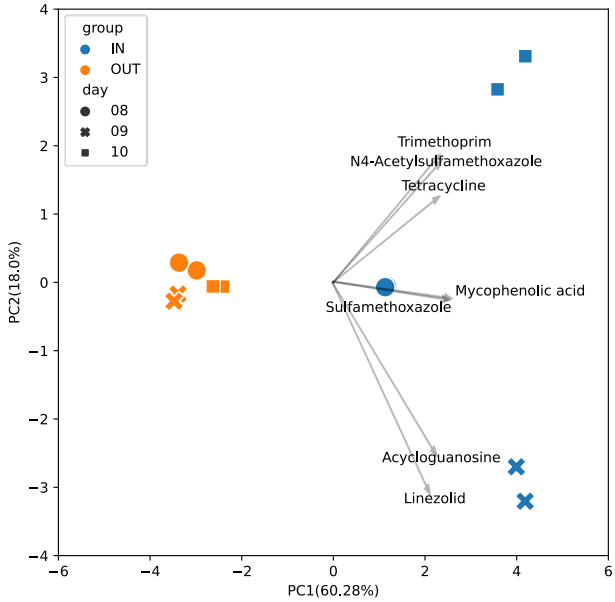

Figure 29. Scatterplot of the first two components of the PCA run on the concentrations of antibiotics in influent and effluent samples (blue and orange color, respectively). The first seven loadings are reported as arrows and annotated with the respective antibiotic. The y value of the loadings was scaled by a factor of 4 for better visualization.

The significance of differences between the concentrations in the two groups (influent and effluent samples) was evaluated by the Mann-Whitney U test. The results shown in Table 14 highlight the fact that the WWTP was able to effectively reduce the concentrations of 11 antibiotics out of the 16 tested (i.e., 2'-Deoxyadenosine, Acycloguanosine, Sulfamethoxazole, N4-Acetylsulfamethoxazole, Clarithromycin, Mycophenolic acid, Linezolid, Tetracycline, Trimethoprim, Clindamycin, and Azithromycin).

Table 14. Results of the Mann-Whitney U test on the concentrations of antibiotics comparing influent and effluent groups. The p-value was corrected for multiple testing with FDR. Only statistically significant results (FDR<0.01) are reported.

Chemical	p-value	FDR-corrected p-value	Fold Change (influent vs effluent)
2'-Deoxyadenosine (Area)	0.00216	0.00590	0.48610
Acycloguanosine (Area)	0.00216	0.00590	84.323
Sulfamethoxazole µg/L	0.00216	0.00590	5.49343
N4-Acetylsulfamethoxazole (Area)	0.00216	0.00590	12.9345
Clarithromycin µg/L	0.00216	0.00590	1.73921
Mycophenolic acid (Area)	0.00277	0.00590	> 84.323
Linezolid (Area)	0.00277	0.00590	> 84.323
Tetracycline µg/L	0.00277	0.00590	> 84.323

Trimethoprim µg/L	0.00499	0.00944	8.5945
Clindamycin µg/L	0.00615	0.00987	0.09887
Azithromycin µg/L	0.00639	0.00987	1.6017

3.2 Biological analysis

The raw reads provided by the sequencing facility were of high quality and sufficient depth. In detail the average number of read pairs was 70.3 M with a standard deviation of 10.23 M ranging from 57.9 to 88.8 M reads, and all average read quality scores were higher than 30.

Only about 1% of reads was discarded in each sample in the trimming process and about 5% of the remaining reads still contained sequencing adapters, which were nevertheless removed. This process resulted in 417.45 M reads in total, which were used for the downstream analyses.

3.2.1 Metagenome-free analysis: bacterial community

The taxonomy assignment and quantification of the samples is reported in Table 15. In the community of all samples the presence of two different kingdoms is observed, i.e., Archea and Bacteria. Starting from the phylum down to the species level, it can be noticed a distinction between influent and effluent samples in terms of OTUs (an index of alpha-diversity), as effluent samples have consistently more OTUs in respect to the influent ones.

Table 15. Results of the taxonomic assignment of the reads based on the QMI-PTDB database. The table reports per sample values and base statistics. Orange lines represent influent samples (08_IN, 09_IN, 10_IN), while light blue lines represent effluent samples (08_OUT, 09_OUT, 10_OUT).

Sample name	Kingdom	Phylum	Class	Order	Family	Genus	Species
08_IN	2	16	16	41	79	253	412
08_OUT	2	30	38	86	153	408	591
09_IN	2	16	16	42	79	254	418
09_OUT	2	25	29	66	132	394	578
10_IN	2	13	14	43	90	315	483
10_OUT	2	28	37	83	156	433	627
Minimum	2	13	14	41	79	253	412
Median	2	20,5	22,5	54,5	111	354,5	530,5
Maximum	2	30	38	86	156	433	627
Mean	2	21,33	25	60,17	114,83	342,83	518,17
Standard deviation	0	7,2	11,06	21,05	36,42	79,66	93,03

The absolute abundances (Figure 30B) illustrate a pronounced distinction between influent and effluent samples. The former exhibits a higher concentration of biologically relevant data. Under a purely quantitative point of view, the effluent samples are characterized by a total number of reads about four times smaller than the influent samples. On the other hand, the relative abundances (Figure 30A) reveal a substantial shift in microbial community composition post-treatment. More specifically, the relative abundance data (Figure 30A) indicate a change from a community dominated by the genera *Acinetobacter* (average 24.8 % of the total reads), *Acidovorax* (16.8%), and *Moraxella* (6.3%) in the influent to a community dominated by *Mycobacterium* (21.8%), *Zooglea* (14.6%), and *Acidovorax* (9.4%) in the effluent. This shift is accompanied by an increased presence of rarer genera. A slight variability is observed in samples of the same group (influent or effluent) sampled on different days; however, it is less marked in respect to the difference between the groups.

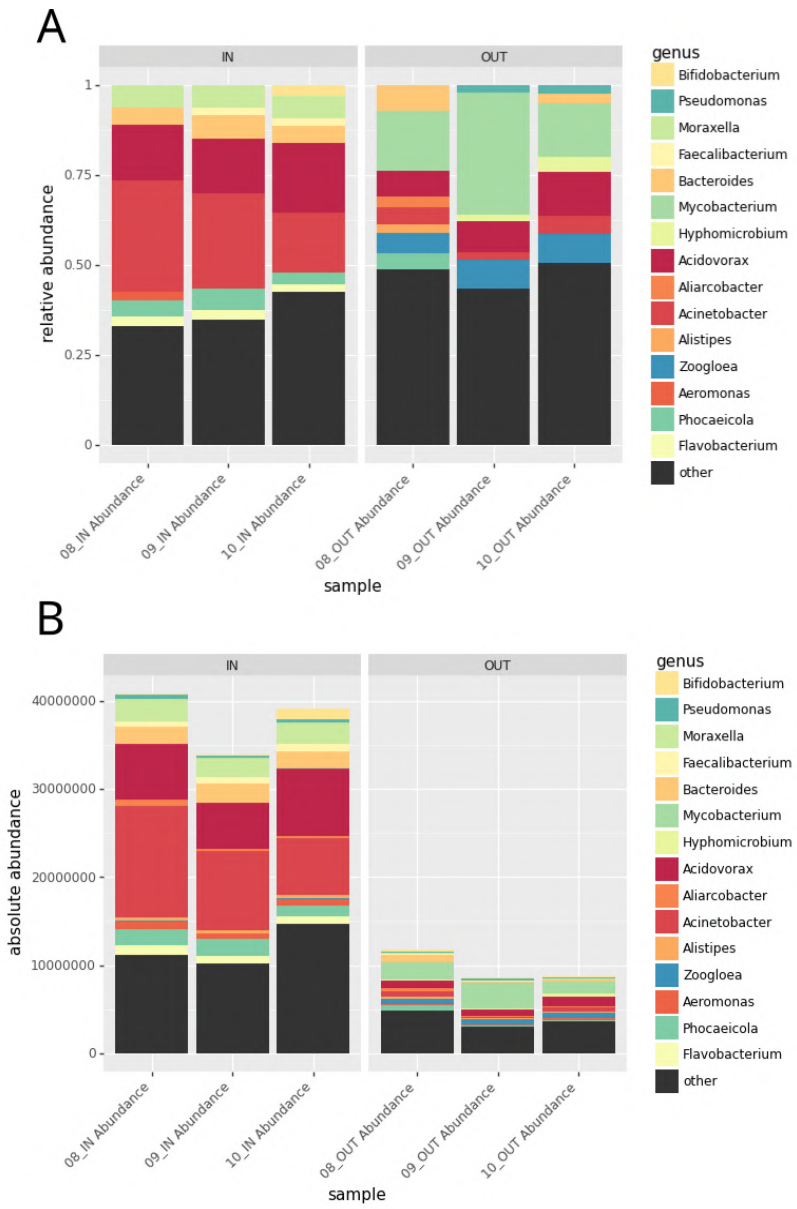

Figure 30. Stacked barcharts of the relative (A) and absolute (B) abundances at the genus level for all the wastewaters samples, grouped by source: influent (IN) and effluent (OUT). Different colors are associated to different genera. Genera with overall relative abundance lower than 0.02 were binned in the "other" group for the sake of visualization.

To further support the previous observations, abundance data were used to compute several key ecological indices: total OTUs, Shannon entropy, Pielou's evenness, and dominance at the family, genus, and species levels (Figure 31). The analysis of the first three metrics revealed consistent trends across these three taxonomic levels, supporting a higher diversity in effluent samples compared to the influent ones. However, a notable increase in dominance was observed at the species level in the influent samples compared to the effluent. This heightened dominance is likely attributable to the species *Acinetobacter johnsonii*, which was the most prevalent species in the influent, accounting for approximately 18% of the total community.

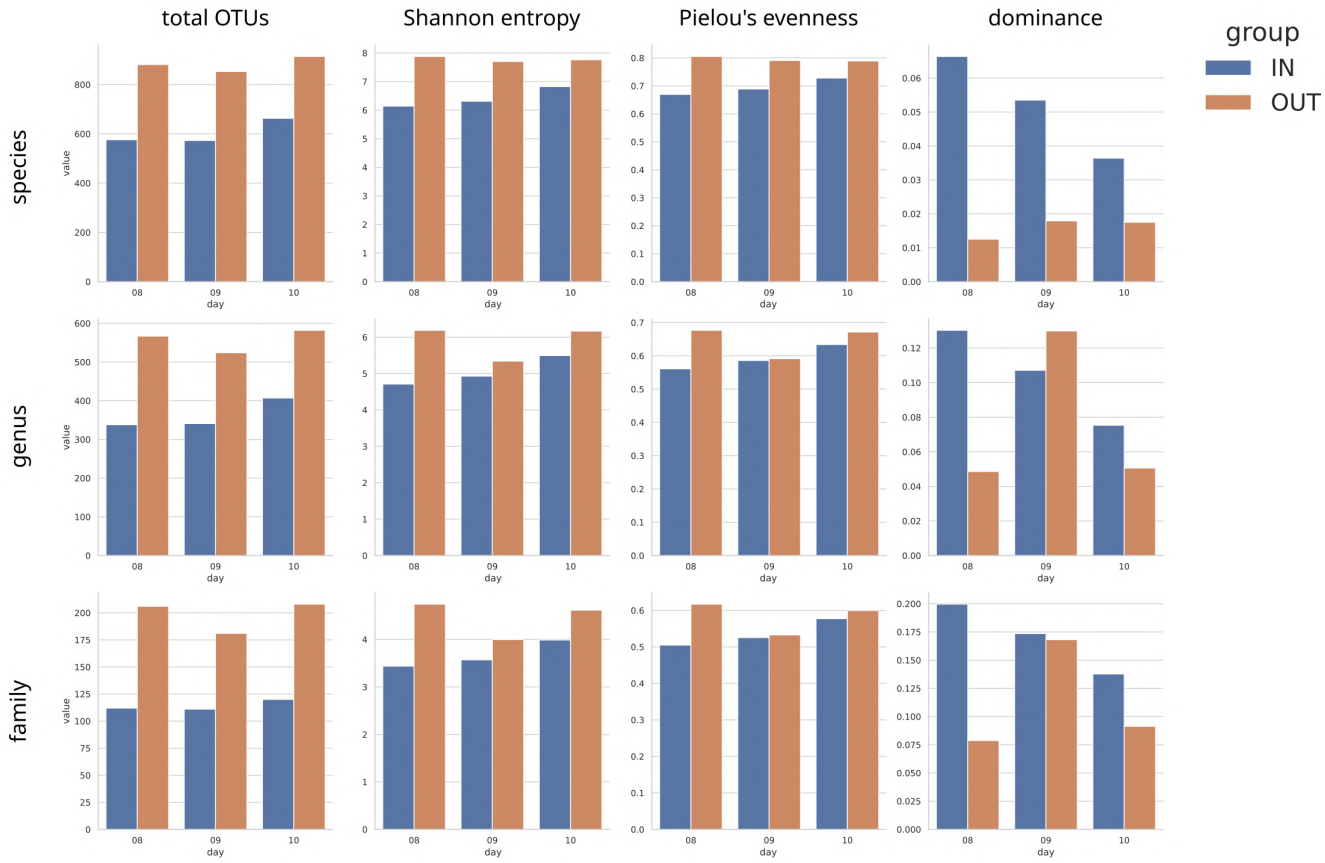

Figure 31. Diversity indices from the metagenome-free analysis: total OTUs, Shannon entropy, Pielou's evenness, and dominance (left to right). Bars are separated by day of sampling and influent samples (IN) are marked in blue color and effluent samples (OUT) in orange color. The four indices are reported by aggregating OTUs at family, genus, and species level (bottom to top).

The three species that most significantly contribute to the differences among the samples are *Acidovorax temperans*, *Moraxella osloensis*, and *Acinetobacter johnsonii* (Figure 32). In the ordination analysis via PCA, a clustering along the Principal Component 1 (PC1) is shown, which appears to be influenced by the source of the samples (influent or effluent waters). The loadings relative to these three species are approximately aligned with the PC1, which alone counts for over 90% of the total variance. This suggests that *Acidovorax temperans*, *Moraxella osloensis*, and *Acinetobacter johnsonii* are the primary drivers of differential abundance among the samples, providing valuable insights into the underlying patterns.

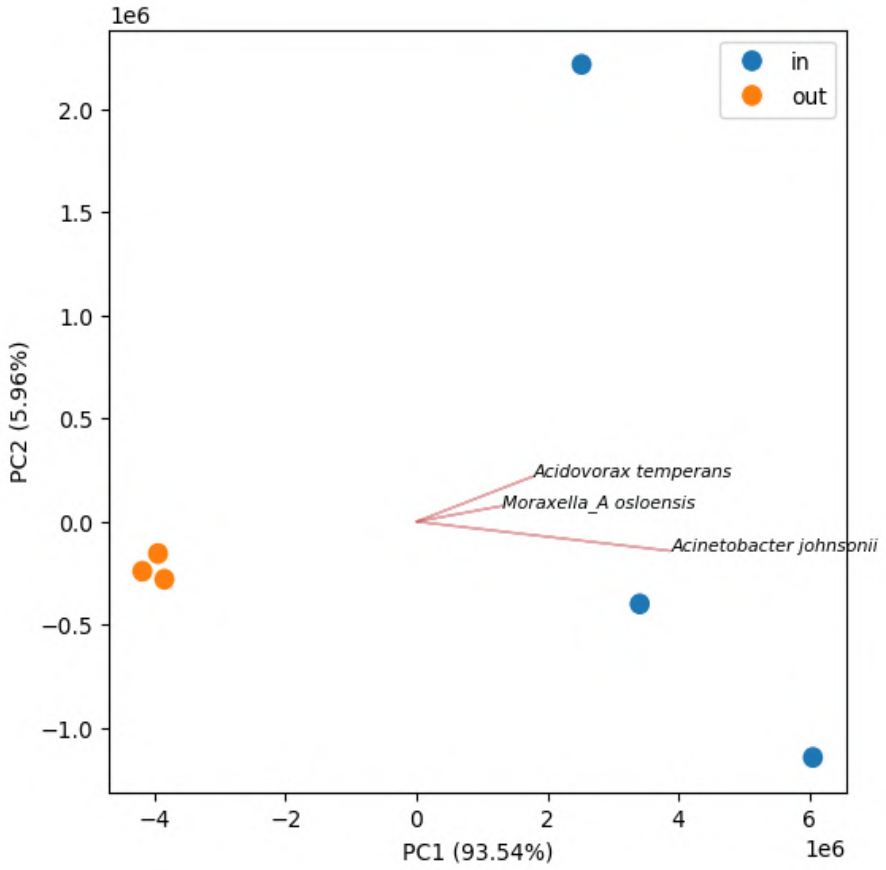

Figure 32. Ordination Plot based on the first two components of the PCA. The top three loadings are shown in the plot. Orange dots refer to effluent water samples, while blue dots refer to influent water samples.

3.2.2 Metagenome-free analysis: antibiotic resistance genes

Counting the ARGs in the samples resulted in a higher representation of resistance genes in the influent waters, relative to the effluent group. A preliminary ordination analysis via PCA showed strong clustering of samples by group, with the first two components explaining more than 99% of the variance (Figure 33). The top 5 loadings (the 5 ARGs with greater weight on this ordination) were represented by: tetracycline-resistant ribosomal protection protein, tet(Q), OXA beta-lactamase, ANT(3'') and msrE.

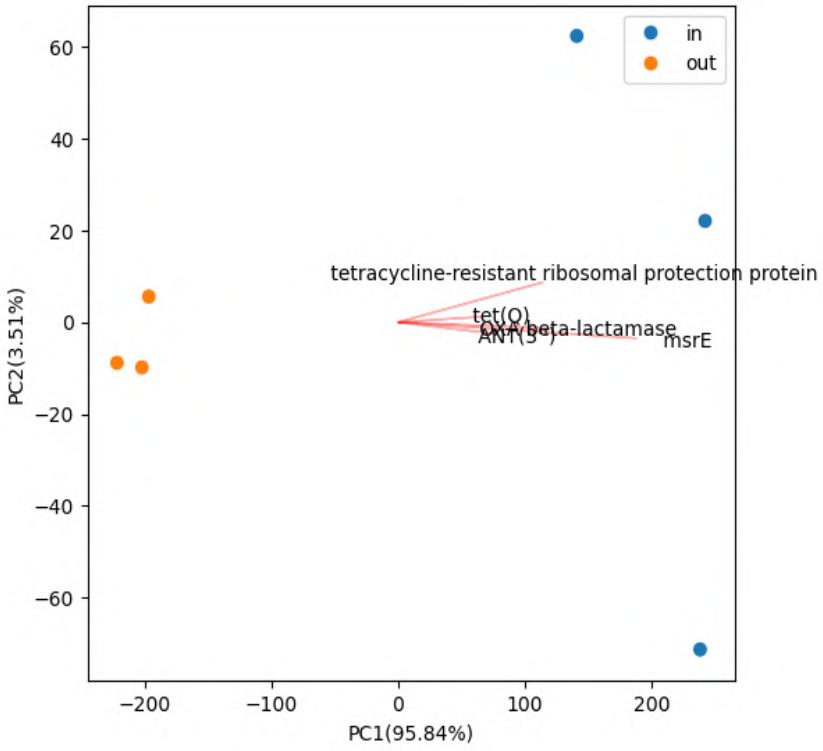

Figure 33. Scatter plot of the first two components of the PCA of the count matrix of ARGs. The top 5 loadings are shown and labelled by the ARG name. Orange dots represent effluent samples, blue dots represent influent samples.

The glm based differential representation analysis between the two groups confirmed this hypotesis, revealing 59 ARGs with significant differential representation (Figure 34). In particular, all but one ARGs were underrepresented in the effluent group, with an exception for the RbpA gene, that was more represented in the effluent group, with a $\log_2$ of Fold Change of +7.79. This increased representation is in line with the taxonomy quantification results, as the RbpA gene is related to the Mycobacterium genus, which is significantly more represented in the effluent samples.

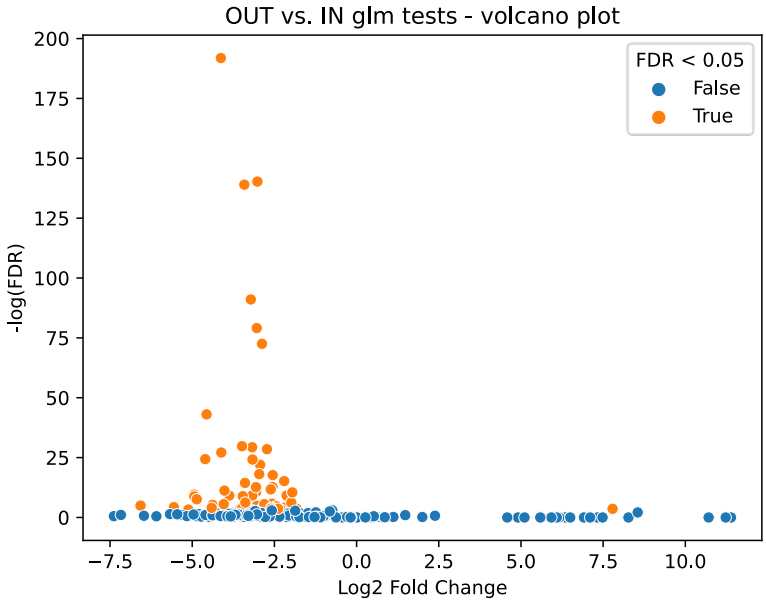

Figure 34. Volcano plot of the differential representation test. The x axis reports the log_2 of fold change in effluent vs. influent group comparison; the y axis reports the significance of the statistical test as -log(FDR). Significantly differentially represented ARGs are reported as orange dots.

A total of 410 ARGs were detected in all sample waters. Of these, 235 ARGs were shared among all 6 samples from both sources (influent and effluents samples). Meanwhile, 155 ARGs were uniquely shared in influent samples and only 20 ARGs in effluent samples (Figure 35).

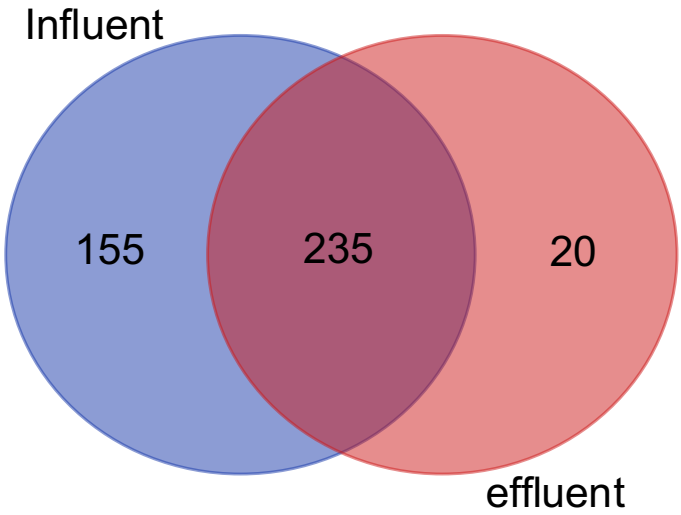

Figure 35. Venn diagram of number of ARGs shared in all six samples (purple color) and ARGs present in influent-only samples (blue color) and effluent-only samples (red color).

The top ten most abundant ARGs in influent samples were: msrE, OXA beta-lactamase, tetracycline-resistant ribosomal protection proteins, ANT(3'')-la, tet(Q), ANT(3'')-la, mphE, ErmF, ErmB, and

CfxA6 (Table 16); whereas OXAbeta-lactamase, tetracycline-resistant ribosomial protection proteins, msrE, ANT(3'')-la, ANT(3''), tet(Q), RbpA, efpA, and majors facilitator superfamily (MFS) antibiotic efflux pump were the top ten observed for effluent samples (Table 17).

Table 16. Most abundant ARGs in influent samples, listed in descending order of abundance, along with their relative resistances to antibiotic classes.

Influent samples			
	ARG	**Normalized abundance**	**Provides resistance to**
	msrE	284,008492	erythromycin and streptogramin B
	OXA beta-lactamase	190,26961	ampicillin and cephalothin
	tetracycline-resistant ribosomal protection protein	188,723861	tetracycline
	ANT(3'')	121,821275	aminoglycoside antibiotics
	tet(Q)	105,763725	tetracycline
	ANT(3'')-Ia	97,713093	aminoglycoside antibiotics
	mphE	66,70658	macrolide antibiotics
	ErmF	38,330629	streptogramin, macrolide, lincosamide
	ErmB	37,444605	erythromycin
	CfxA6	37,394192	cephamycin

Table 17. Most abundant ARGs in effluent samples, listed in descending order of abundance, along with their relative resistances to antibiotic classes.

Effluent samples			
	ARG	**Normalized abundance**	**Provides resistance to**
	OXA beta-lactamase	23,485681	ampicillin and cephalothin
	tetracycline-resistant ribosomal protection proteins	17,685937	tetracycline
	msrE	16,23447	erythromycin and streptogramin B
	ANT(3'')-Ia	13,300667	aminoglycoside antibiotics
	ANT(3'')	13,099114	aminoglycoside antibiotics
	tet(Q)	12,894208	tetracycline
	RbpA	12,447322	rifamycin
	efpA	8,511239	isoniazid-like antibiotic, rifamycin
	major facilitator superfamily (MFS) antibiotic efflux pump	7,709331	tetracycline, chlorampenicol, fluoroquinolones, macrolides, rifampin, fosfomycin
	OXA-46	5,650731	OXA beta-lactamase

A differential representation test based on a generalized linear model (GLM) was employed to evaluate the changes in ARGs prevalence between influent and effluent waters. This analysis showed that, despite having a shared resistome in both influent and effluent samples, a significant reduction in ARGs quantities is observed, as the most abundant ARGs in the influent were 8~16-fold diminished in the effluent (Table 18).

Table 18. Top ten most significantly differentially represented ARGs in the comparison between effluent and influent samples obtained by the GLM.

Gene	p-value	FDR	Log_2 Fold Change	provides resistance to
msrE	4.75E-84	4.75E-84	-4.128802	erythromycin and streptogramin B
OXA beta-lactamase	1.19E-61	1.19E-61	-3.018192	ampicillin and cephalothin
tetracycline-resistant ribosomal protection protein	4.34E-61	4.34E-61	-3.415602	tetracycline
ANT(3'')	2.89E-40	2.89E-40	-3.217225	aminoglycoside antibiotics
tet(Q)	4.47E-35	4.47E-35	-3.03605	tetracycline
ANT(3'')-Ia	3.14E-32	3.14E-32	-2.877053	aminoglycoside antibiotics
mphE	2.00E-19	2.00E-19	-4.563996	macrolide antibiotics
ErmF	1.20E-13	1.20E-13	-3.485048	streptogramin, macrolide, lincosamide
CfxA6	1.85E-13	1.85E-13	-3.177264	cephamycin
OXA-46	4.14E-13	4.14E-13	-2.72593	OXA beta-lactamase

3.2.3 Metagenome-free analysis: co-occurrence network analysis

Co-occurrence networks consisting of nodes and edges representing genera-ARGs and genera-genera relationships were generated for both influent and effluent water samples. In each source, ARGs and microorganisms that reach a correlation coefficient threshold were linked with lines to visualize correlation patterns and evaluate potential hosts for ARGs [174]. Both genera-genera co-occurrence networks (Figure 36B and Figure 37B) exhibited modularity indices greater than 0.4: 0.51 for the influent and 0.67 for the effluent. A similar trend was observed in the genera-ARGs networks (Figure 36A and Figure 37A), where the modularity value was 0.41 in influent samples and 0.60 in effluent samples. High modularity index values indicate that these networks exhibit a well-defined modular structure rather than a random configuration. This observation can be interpreted as evidence of a robust community structure comprising modules, hubs, and clusters [185].

The influent and effluent networks consisted of 272 and 201 total nodes, respectively, of which 138 and 54 were ARG nodes. Additionally, the influent genera-ARGs network displayed a higher number of edges (interactions), with 13,184 compared to the effluent genera-ARGs network, which had 5,141 edges. However, when ARG nodes were removed (genera-genera networks), the edge values changed to 1,932 for the influent and 2,000 for the effluent. These differences may suggest variations in the strength of interactions between nodes (genera and/or ARGs) in each source. A summary of nodes, edges, and modularity values for the genera-genera and genera-ARGs co-occurrence networks is presented in Table 19.

Table 19. Network metrics: nodes, edges, and modularity values of genera-genera (orange color) and genera-ARGs (light blue color) co-occurrence networks of both influent and effluent sources.

	INFLUENT genera-genera	**EFFLUENT genera-genera**	**INFLUENT Genera-ARGs**	**EFFLUENT Genera-ARGs**
Nodes	138	147	272	201
Edges	1,932	2,000	13,184	5,141
Modularity	0.51	0.67	0.41	0.60

In the influent samples, the co-occurrence meta-network of genera-ARGs interactions (Figure 36A) revealed three major clusters, each containing a distinct set of ARGs acting as hubs. Notably, the ARG VIM-7 was central to two of these clusters, showing high co-occurrence with many other ARGs. When considering only the co-occurrences among genera (Figure 36B), the network was composed of eight independent clusters, with one cluster appearing more denser than the others, with a modularity of 0.51.

In contrast, the effluent samples exhibited a more complex network structure in both genera-ARGs and genera-genera networks. Similar to the influent network, the genera-ARGs co-occurrence network for the effluent (Figure 37A) could also be divided into three modules, each containing central ARG hub clusters. However, the overall network structure appeared more complex. The genera-genera co-occurrence network (Figure 37B) consisted of two discrete clusters, each containing well-defined subclusters. Within these subclusters, the genera *Microthrix*, *Pararhodobacter*, and an unidentified genus belonging to the Pseudonocardiaceae family served as key hub nodes. This network exhibited a modularity of 0.67.

It is important to note that, for both the influent and effluent networks, the modularity values increased once the ARG-derived nodes were removed: from 0.411 to 0.51 in influent waters; from 0.60 to 0.67 in effluent waters.

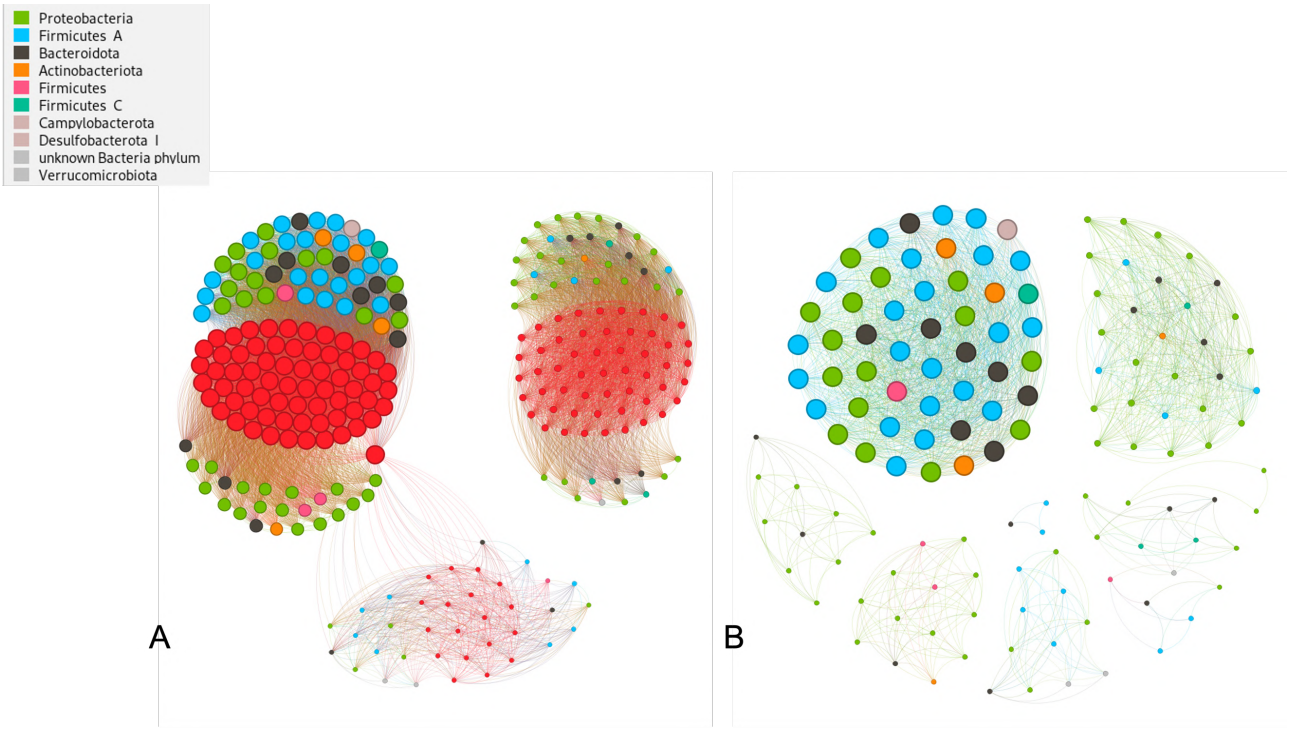

Figure 36. Co-occurrence network showing the correlations between genera-ARGs (A) and genera-genera (B) in the influent samples. ARGs are represented by red nodes (in A), any other colored node represents a genus, colored by phylum, and scaled by eigenvector centrality. The size of the nodes is proportioned to eigenvector degree, while the width of lines between nodes is proportioned to the correlation coefficient of Spearman's ρ rho-value.

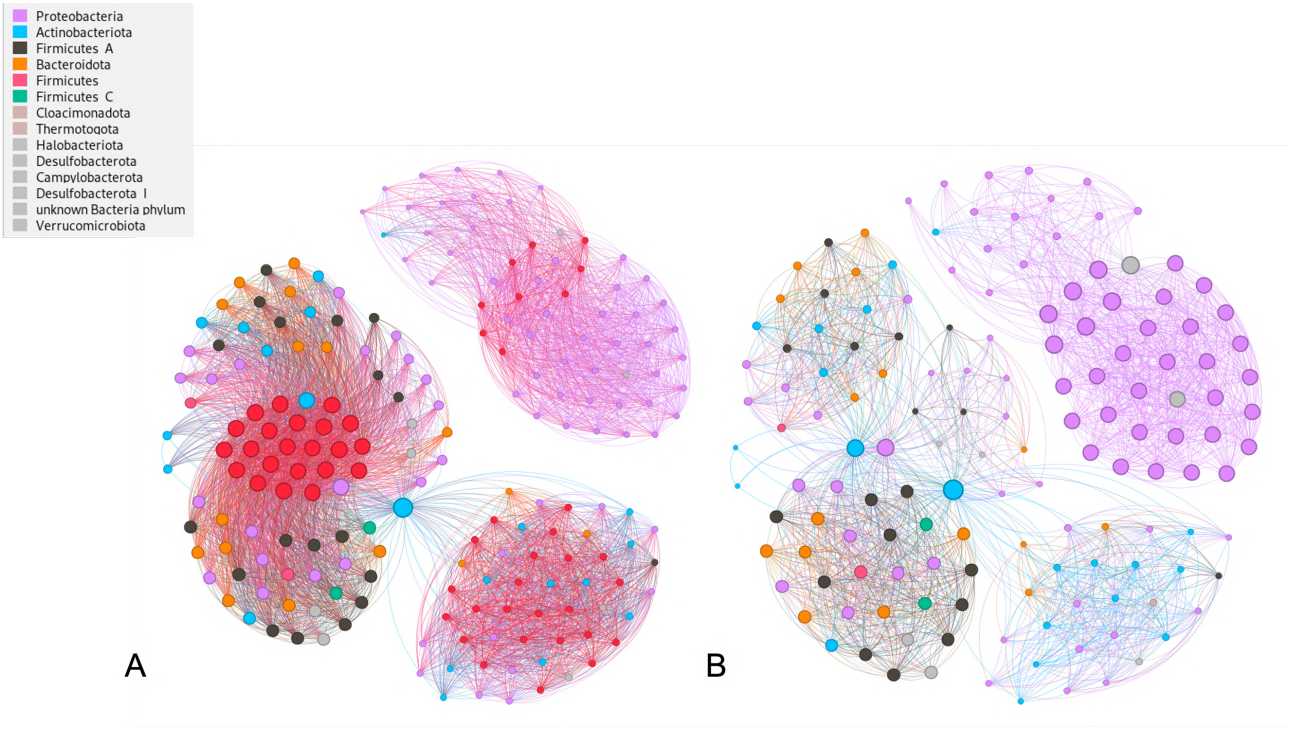

Figure 37. Co-occurrence network showing the correlations between genera-ARGs (A) and genera-genera (B) in the effluent samples. ARGs are represented by red nodes (in A), any other colored node represents a genus, colored by phylum, and scaled by eigenvector

centrality. The size of the nodes is proportioned to eigenvector degree, while the width of lines between nodes is proportioned to the correlation coefficient of Spearman's ρ rho-value.

3.2.4 Metagenome-based analysis

The evaluation of multiple assembly strategies was conducted to identify the most appropriate approach, focusing on metrics such as contiguity, completeness, and computational efficiency. Key performance metrics of the different assembly methods are briefly compared in the following sections.

Contiguity was assessed by calculating the Nx metric for $1 < x < 18$ and plotting these values alongside the classical N50 (Figure 38). Together, these metrics complement each other by offering both granular and holistic perspectives on assembly contiguity. The results indicate that co-assemblies generally produce more contiguous outcomes than individual sample assemblies. Both assemblers demonstrated adequate performance under this criterion; however, megaHit exhibited consistently better results compared to metaSPAdes.

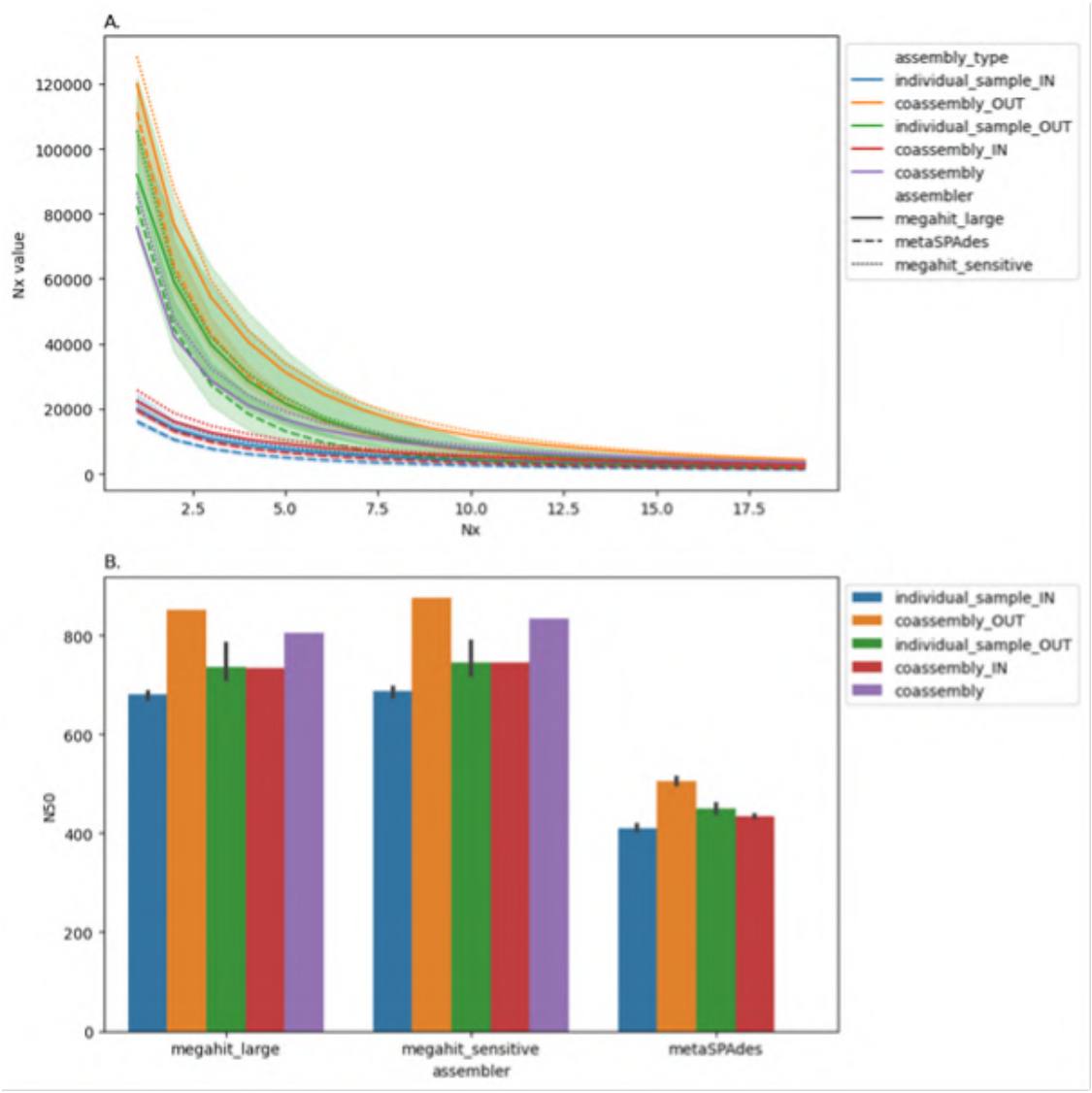

Figure 38. Nx plot

This trend is further supported by the total number of assembled sequences (Figure 39): megaHit assemblies yielded fewer sequences, indicating reduced fragmentation. When considered in conjunction with the higher N50 values, the megaHit assemblies appear more contiguous, a finding that is also substantiated by the distribution of contig sizes (Figure 40).

These observations, combined with the inability of metaSPAdes to generate a co-assembly for all samples, demonstrate that the megaHit-based co-assembly (with the sensitive flag mode) is the most suitable reference for downstream analyses. Moreover, employing a single, comprehensive assembly as the reference framework simplifies comparative analyses across samples.

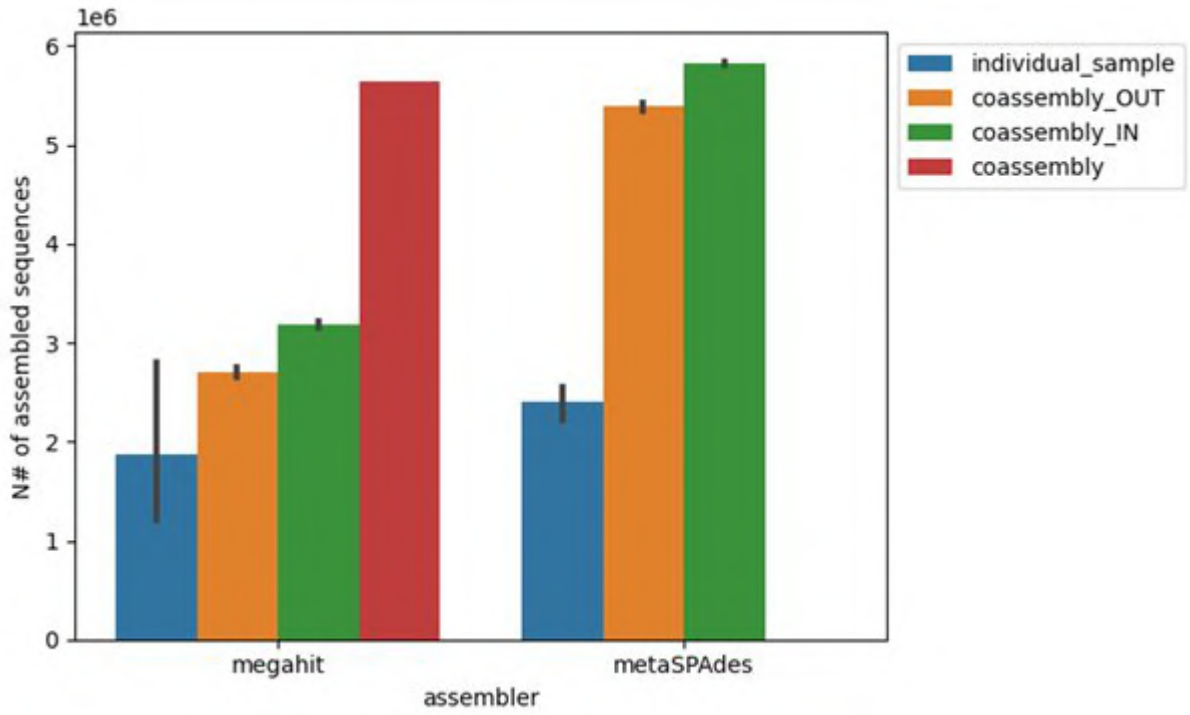

Figure 39. Number of assembled sequences per assembly.

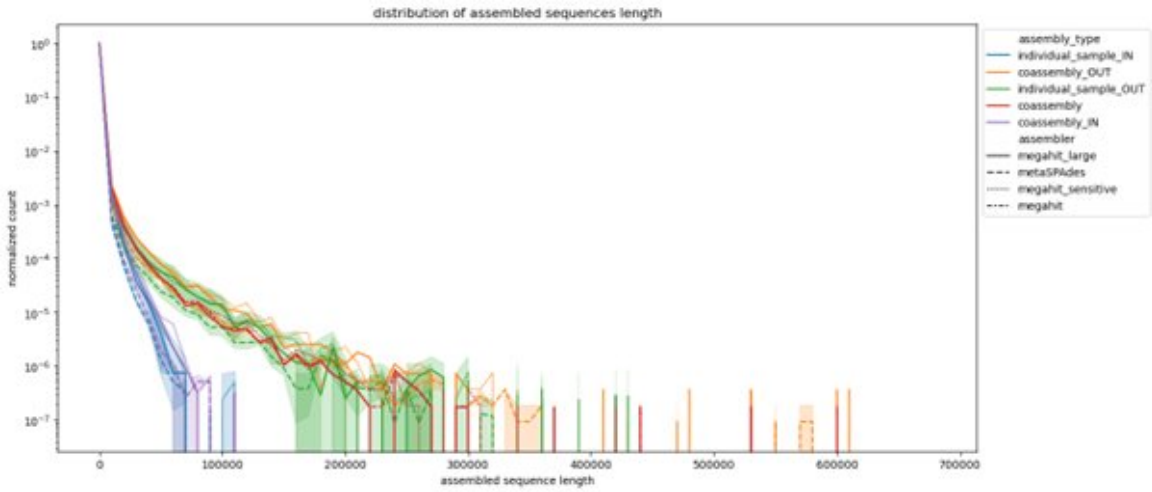

Figure 40. Assembled sequence length for the various assembly approaches.

The final co-assembly consisted of 3.219.995 assembled contigs, for a total size of approximately 2.12Gb and no ambiguous nucleotides. L50 and N50 values were respectively 993.037 and 808, which indicate a very fragmented assembly but might be expected given the very dirty origins of our samples. The same values calculated by removing the sequences shorter than 1kb were 133.905 and 2.812, indicating that most of the background noise is given by very short sequences.

The GC content distribution (Figure 41) exhibited two main peaks, around 40% and 65%, along with less pronounced peaks, indicating a degree of compositional diversity in the assembled contigs, which aligns with expectations for heterogeneous samples such as sewage water.

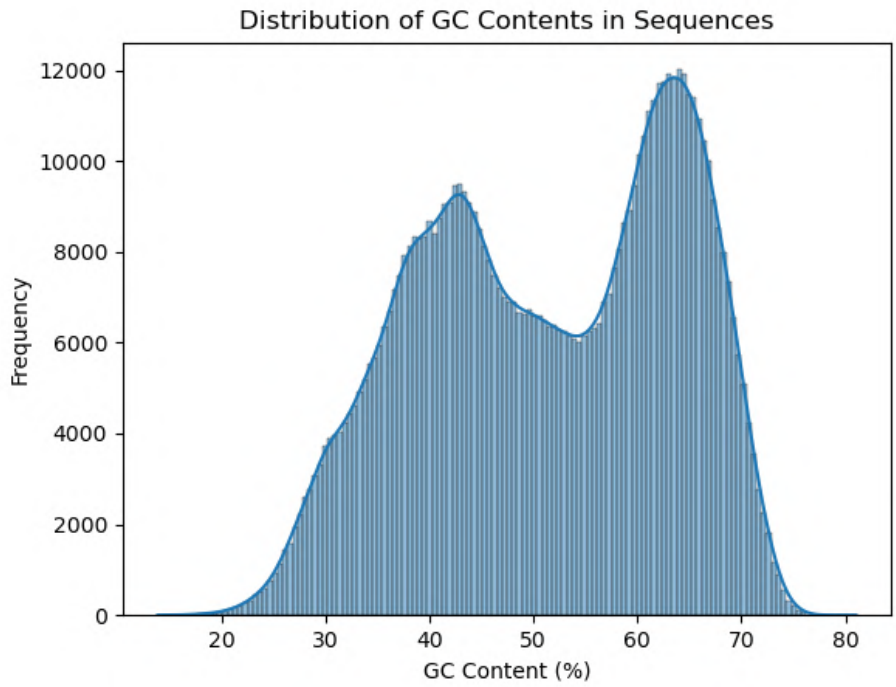

Figure 41. Distribution of GC content in the assembled metagenome.

The iterative binning process, based on three software, filtering, reassembly and annotation produced a total of 199 bins, averaging 372.71 sequences, with an average size of 2.19Mb and a mean GC content of 50.72%. In particular, the distribution of the GC content closely reflected that of the overall metagenome assembly, displaying the two main peaks around 45% and 65% (Figure 7).

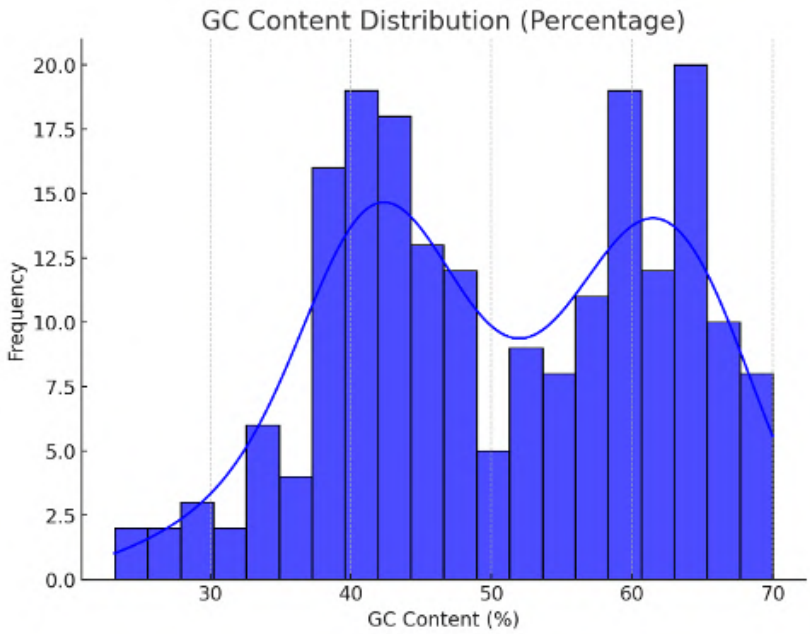

Figure 42. Distribution of GC percentage sequence composition in the binned MAGs.

4 Discussion

A multidisciplinary approach has been designed to analyze and profile antibiotics in influent and effluent waters at the WWTP in Trieste. Initial findings highlight the presence and behavior of various antibiotic substances, offering valuable insights into their dynamics within the treatment process.

4.1 Chemical analysis

The mass of antibiotic substances entering the sewer system is strongly influenced by human behavior, including the improper use or disposal of pharmaceuticals. Additional contributing factors include the number of prescriptions issued based on medical diagnoses, variations in drug metabolism within the human body (drug-dependent metabolism), and any pre-treatment of wastewater, which is typically performed when the drug is administered in a hospital settings [82].

The study on antibiotic drugs was conducted to identify and assess the concentrations of antibiotic drugs and their metabolites, which exert selective pressure on bacteria present in wastewaters. It is known that this pressure promotes the survival, proliferation, and, consequently, the emergence of pathogenic bacteria with antibiotic-resistant capabilities [84,87].

The z-score concentrations show an effective abatement of most molecules examined in this study, such as acycloguanosine, clarithromycin, the metabolite N-desmethyl clarithromycin, mycophenolic acid, linezolid, tetracycline, trimethoprim, azithromycin, sulfapyridine, sulfamethoxazole and the metabolite N4-acetylsulfamethoxazole.

Acycloguanosine, also known as acyclovir, is an antiviral medication primarily used to treat infections caused by herpesviruses [186]. This compound is frequently detected in hospital and domestic wastewaters due to its widespread use. The resistance of acycloguanosine to conventional wastewater treatment processes is compounded by its poor metabolism in humans, leading to excretion of both the parent compound and its metabolites [187].

Clarithromycin, a macrolide antibiotic, is widely used to treat bacterial infections, particularly those affecting the respiratory tract, skin, and soft tissues. As a semisynthetic derivative of erythromycin, it offers improved pharmacokinetic properties compared to its parent compound [188]. Frequently detected in WWTPs, clarithromycin has been linked to increasing resistance rates among *Helicobacter pylori* and respiratory pathogens like *Streptococcus pneumoniae* and *Haemophilus influenzae* [188–192].

N-desmethyl clarithromycin is a primary metabolite of clarithromycin and retains antibacterial activity. However, it is less effective against many pathogens compared to clarithromycin or its other major metabolite, 14-hydroxy clarithromycin. N-desmethyl clarithromycin is classified as an emerging contaminant due to its potential environmental and ecological impacts [193]. Studies have identified this metabolite in both raw and treated waters within WWTPs due to its recalcitrant properties. Conventional treatment methods fail to degrade N-desmethyl clarithromycin effectively because of its resistance to microbial degradation and low reactivity under standard oxidative processes. Advanced treatment methods, such as reverse osmosis, are effective because they physically separate contaminants regardless of chemical structure, providing a more reliable removal mechanism [194,195]. The presence of antibiotic metabolites like N-desmethyl clarithromycin in the environment can contribute to the development of antibiotic-resistant bacteria, posing risks to both ecosystems and human health [196].

Mycophenolic acid (MPA) is a secondary metabolite with potent immunosuppressive and antimicrobial properties. Primarily used in medicine as part of immunosuppressive therapy to prevent organ rejection after transplantation, MPA exhibits antifungal, antibacterial, and antiviral properties [197]. It is detected in wastewater effluents, particularly from pharmaceutical manufacturing plants or hospitals using immunosuppressive drugs [198]. The prolonged exposure of microbial communities to MPA in the environment could select for resistant strains [199].

Linezolid, a synthetic antibiotic belonging to the oxazolidinone class, is primarily used to treat serious infections resistant to other antibiotics, such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE) [200]. Although resistance to linezolid remains relatively rare, its increased use, particularly in healthcare settings, is driving the emergence of resistant strains of E*nterococcus faecium* and *S. aureus* with ***cfr* genes** that exhibit cross-resistance to other oxazolidinone [201–203]. Detected in hospital and municipal wastewater due to incomplete metabolism and excretion, the stability of linezolid as a molecule contributes to the selection of antibiotic-resistant bacteria in aquatic environments [204].

Tetracycline is a broad-spectrum antibiotic belonging to the tetracycline class and has been widely used to treat bacterial infections since its discovery in the 1940s, for both human and veterinary medicine [205]. Commonly detected alongside with its metabolites in agricultural runoff, hospital effluents, and municipal wastewaters [142], tetracyclines are notably persistent in the environment. This persistence significantly contributes to the development of ARGs in microbial communities, particularly *E. coli* and *S. aureus* [142,206,207].

Trimethoprim is a synthetic antibiotic primarily used to treat bacterial infections and is often combined with sulfamethoxazole (under the brand name co-trimoxazole) to enhance its efficacy [208]. Commonly detected in municipal wastewater and hospital effluents, the widespread use and incomplete metabolism of trimethoprim contribute to its environmental presence [209]. In addition, its increasing use, particularly for treating urinary tract infections, has raised concerns over antibiotic resistance. Resistance to trimethoprim develops through mutations in genes such as *folA*, dfrA1, dfrA5, and dfsrB in bacteria like *E. coli*, *S. aureus* , and *S. pneumoniae* [210–212].

Azithromycin is a macrolide antibiotic widely used to treat various bacterial infections, including respiratory and skin diseases [213]. Frequently detected in hospital and municipal wastewaters due to incomplete metabolism and excretion [191], azithromycin remains stable in the environment, contributing to the selection of antibiotic-resistant bacteria in aquatic ecosystems. Resistance has been observed in bacteria such as *S. pneumoniae*, *S. aureus*, *Neisseria gonorrhoeae*, *Campylobacter jejuni*, and *Campylobacter coli* [214–218].

Sulfapyridine is an antibacterial compound belonging to the sulfonamide class of medications, commonly known as sulfa drugs. It is one of the most frequently detected sulfonamides in various environmental systems such as wastewater effluents and receiving water bodies [219].

Sulfamethoxazole (SMX) is a synthetic antibiotic belonging to the sulfonamide class. Commonly used in combination with trimethoprim under the brand name co-trimoxazole (Bactrim), this combination exhibits broad-spectrum antibacterial activity effective against various bacterial infections, including *E. coli*, *Klebsiella pneumoniae*, *S. aureus*, and *S. pneumoniae*. Due to its

widespread use and incomplete metabolism in humans [220], SMX is frequently detected in wastewaters. It is relatively resistant to conventional treatment processes in WWTPs [221], and its prolonged environmental exposure can contribute to the development of ARGs in bacteria [222]. Although some sulfamethoxazole may degrade during treatment, its apparent concentration often remains stable due to its regeneration from N4-acetylsulfamethoxazole by deacetylation [223].
N4-Acetylsulfamethoxazole is a major metabolite of the sulfonamide antibiotic SMX. Despite being an inactive metabolite, it is relevant in both clinical pharmacology and environmental studies. Primarily excreted in urine, this metabolite serves as a marker of SMX metabolism and dosing. It is commonly detected in the wastewater environment, and it is usually found in effluents due to its resistance to conventional wastewater treatment processes. The study of N4-Acetylsulfamethoxazole is significant because it can mask the removal efficiency of SMX as it may be converted back into this parent compound [223,224]. Its persistence in the environment and potential role in antibiotic resistance emphasize the need for improved wastewater treatment technologies and monitoring.
Since most of these antibiotics are reported in the literature to be resistant to treatment processes in WWTPs, it can be concluded that the WWTP of Trieste is particularly efficient at removing these drugs from wastewaters.

The z-score concentrations of antibiotics show negligible changes after the plant treatment processes for the following four antibiotics: fluconazole, ciprofloxacin, ethambutol, and levofloxacin.
This is likely due to their chemical stability or resistance to biodegradation based on their chemical structures, which make them resistant to conventional degradation mechanisms such as hydrolysis or biodegradation [92]. This process is influenced not only by the initial concentration of antibiotics in the influent wastewater but also by environmental conditions or geographic locations. For instance, warmer climates with higher temperatures and greater solar radiation could enhance photodegradation and chemical breakdown, while cooler regions may exhibit slower degradation rates. Similarly, the composition and activity of microbial communities, which differ by region, can substantially influence the biological degradation processes of these compounds [93,94]. These findings highlight the limitations of current wastewater treatment strategies and suggest the need for advanced treatment technologies capable of addressing such persistent compounds.
Fluconazole is a triazole antifungal medication widely used to treat and prevent various fungal infections. The emergence of fluconazole-resistant fungal strains (like *Candida albicans*) poses significant clinical challenges [225]. Conventional WWTPs often exhibit limited efficiency in removing such pharmaceuticals, potentially due to the molecular properties of compounds like fluconazole, which are highly stable and resistant to degradation, making them persist in wastewater

and potentially accumulate in aquatic environments. Additionally, process limitations, such as inadequate retention times or lack of advanced treatment technologies, contribute to their release into surface waters [225].

Ciprofloxacin is a broad-spectrum antibiotic belonging to the fluoroquinolone class, and it is widely used to treat a variety of bacterial infections, particularly those caused by Gram-negative organisms [226]. Its presence in aquatic environments can exert selective pressure on microbial communities, promoting the development and dissemination of ARGs. Resistance is particularly high among *E. coli* isolates in urinary tract infections and hospital-acquired infections caused by *Pseudomonas aeruginosa* [227]. Ciprofloxacin is frequently detected in hospital and municipal wastewaters due to its widespread use and incomplete metabolism in humans. Studies have reported concentrations ranging from nanograms to micrograms per liter in influents and effluents [228,229].

Ethambutol is an antimycobacterial agent primarily used in the treatment of tuberculosis (TB), and its widespread use contributes to its environmental persistence, raising concerns about its potential accumulation in wastewater systems. Ethambutol is effective against *Mycobacterium tuberculosis* strains but less effective against viruses, fungi, or other bacteria [230]. Resistance to ethambutol can develop through mutations in the embB gene, which encodes arabinosyl transferase [231]. A study aimed to optimize analytical methods for detecting TB drugs, including ethambutol, in treated and untreated wastewater across several African countries, showed that ethambutol was consistently the most detected drug, with concentrations in treated wastewater reaching up to 10.20 ng/mL [232]. Such high concentrations raise significant concerns about potential public health risks, including the promotion of antimicrobial resistance, and environmental risks, such as bioaccumulation in aquatic organisms.

Levofloxacin is a broad-spectrum fluoroquinolone antibiotic used to treat a variety of bacterial infections, including pneumonia, urinary tract infections, skin infections, and acute bacterial sinusitis [233]. Studies have reported levofloxacin concentrations ranging from nanograms per liter (ng/L) to micrograms per liter (μg/L) in treated and untreated wastewaters [191]. Studies from countries like South Korea, Canada, the USA, and Spain demonstrate a correlation between increased fluoroquinolone use, including levofloxacin, and the development of microbial resistance [234]. Resistance has been observed in hospital-acquired infections, with reduced effectiveness in treating pneumonia and documented treatment failures in *S. pneumoniae* [235].

In contrast, clindamycin and 2’-deoxyadenosine increase in concentration in effluent samples in respect to the influent ones. This may be attributed to transformation processes or the release of conjugated forms during the treatment processes of the WWTP [223].

Clindamycin is a lincosamide antibiotic effective against various bacterial infections, particularly those caused by Gram-positive aerobes and anaerobes [236]. Studies have reported varying rates of inducible clindamycin resistance among *S. aureus* and MRSA [237,238]. Clindamycin exhibits low biodegradability and high chemical stability, leading to its persistence through conventional wastewater treatment processes. Studies have reported that clindamycin is one of the most frequently detected antibiotics in effluent waters after the WWTP, indicating its resistance to standard treatment methods [239,240]. Clindamycin can be otherwise formed in WWTPs through the biotransformation of its metabolites or conjugates. For instance, clindamycin phosphate, a prodrug, may undergo dephosphorylation, regenerating active clindamycin. Additionally, conjugated forms of clindamycin can be hydrolyzed during treatment, releasing the parent compound [241]. In addition, due to its hydrophobicity, clindamycin can adsorb onto sludge during treatment and later desorb back into the aqueous phase, leading to higher concentrations in the effluent waters. This behavior complicates its removal and contributes to its presence in treated wastewaters [241].

2'-Deoxyadenosine is a naturally occurring nucleoside composed of the purine base adenine attached to a deoxyribose sugar. It is an important building block of DNA, where it forms part of the nucleotide deoxyadenosine monophosphate (dAMP). As a degradation product of DNA or nucleoside-based drugs, 2'-deoxyadenosine may be detected in antibiotic-contaminated environments such as hospital effluents or biotechnological waste. The presence of 2'-deoxyadenosine in these settings can pose risks to environmental and public health, as its persistence may contribute to the proliferation of ARGs and alter microbial ecosystems. Microbial activity within WWTPs can lead to the release of 2'-deoxyadenosine through the breakdown of microbial biomass. In particular, enzymatic degradation of nucleic acids during treatment processes can increase nucleoside concentrations in the effluent. Processes such as microbial lysis release nucleic acids, which are subsequently broken down into nucleosides like 2'-deoxyadenosine. [242–245].

Both clindamycin and 2'-deoxyadenosine may resist degradation in WWTPs due to their chemical structures leading to recalcitrance to the treatment processes. Clindamycin, for example, has been found to persist in surface waters even after treatment, suggesting that conventional biological processes are insufficient for its complete removal. This resistance can be attributed to its stable chemical structure, including its chlorine-substituted thioether group, which makes it less susceptible to biodegradation and other treatment mechanisms. However, intermittent discharges from hospitals or pharmaceutical industries can introduce additional loads of clindamycin into the WWTP, resulting in elevated concentrations in the effluent. Such sources may not be consistent, leading to fluctuations in influent and effluent concentrations [240].

In the PCA plot run on the concentrations of antibiotics in influent and effluent samples, the mycophenolic acid, N4-acetylsulfamethoxazole, sulfamethoxazole, trimethoprim, and tetracycline are the top five antibiotics with the major loading. This indicates that these compounds are primary contributors to the variability captured by the PCA, potentially due to their significant abundance or distinct chemical behavior. Understanding their major loading is crucial to guide targeted interventions for their removal. The cluster based on the sampling source indicates that this variable plays a major role in driving variability within the dataset, potentially reflecting distinct environmental conditions associated with each source. These conditions may include variations in the microbial community composition, chemical characteristics of the wastewater, or external factors such as local temperature and industrial discharge patterns. On the other hand, the cluster based on the day of sampling suggests potential temporal variability in the dataset, which may be influenced by factors such as weather changes, or variations in input characteristics over time.

These findings contribute to broader strategies for managing antibiotic resistance by highlighting the need to mitigate the release of antibiotics into wastewater systems. Implementing advanced treatment technologies and promoting responsible use and disposal of antibiotics could help reduce the selective pressure driving the proliferation of antibiotic-resistant bacteria. Implementing advanced wastewater treatment technologies, such as ozonation or activated carbon adsorption, could enhance the removal of persistent compounds. Additionally, promoting public awareness campaigns on proper disposal of pharmaceuticals and enforcing stricter regulations on industrial and hospital discharges could significantly reduce environmental contamination and limit the spread of antibiotic resistance.

4.2 Biological analysis

The introduction of metagenomics approaches allows for PCR-independent assessment for molecular investigation of biological activities within an environmental community [246]. The WWTPs are designed to filter biohazard residuals and reduce the microbial load (in particular pathogenic organisms), both physically and chemically, within different treatment stages [247]. The nature of the wastewater treatment with a high concentration of bacterial communities increases the possibility of acting as a reservoir for gene transfer (e.g., HGT). This can facilitate the spread of ARGs, which pose significant risks, including the potential to compromise the efficacy of antibiotics in clinical settings and disrupt ecological balance by altering microbial community functions [107,129].

Within this study, samples of influent and effluent water were collected at the WWTP of Trieste (Italy) to analyze changes in the microbial community and the presence of ARGs before and after the treatment processes of the plant. A metagenomic approach was employed to identify and quantify microbial taxa and ARGs, providing a comprehensive overview of the impact of treatment processes. In particular, assessing the intrinsic and extrinsic resistomes from samples would provide a deeper understanding to confirm or dispute the hypothesis that a WWTP may act as a reservoir for horizontal gene transfer. This process poses a risk of altering ARG distribution patterns, potentially leading to broader environmental dissemination [248].

4.2.1 Metagenome-free analysis: bacterial community

Quantitatively, effluent samples exhibit a total number of reads approximately four times smaller than influent samples. This difference highlights the impact of the treatment process in reducing microbial biomass, which could indicate effective removal of microorganisms. A pronounced distinction between influent and effluent samples is illustrated in the absolute abundances (Figure 30B). The influent water exhibits a higher concentration of biologically relevant data. This observation aligns with expectations, considering the higher density of influent waters compared to the effluent ones, thereby indicating again the efficacy of the WWTP in reducing the influent biomass [85].

Moreover, the relative abundances (Figure 30A) reveal a substantial shift in microbial community composition after the treatments of the WWTP. *Acinetobacter*, *Acidovorax* and *Moraxella* are the dominant genera in influent samples. In contrast, *Mycobacterium*, *Zooglea* and again *Acidovorax* (but with a lower dominance compared to influent samples) are the most abundant genera in the effluent samples. The observed change in dominant bacterial genera from influent to effluent samples in this study may reflect the selective pressures and environmental conditions linked to wastewater treatment processes. Species within *Acinetobacter* genus are prevalent in influent wastewaters probably due to their metabolic versatility, enabling them to degrade a wide range of organic compounds, such as hydrocarbons, fatty acids, and aromatic compounds [46]. This adaptability allows to dominate in complex nutrient environments typical of influent waters. Additionally, these species possess diverse physiological characteristics associated with some important microbiological aspects, such as biofilm production, quorum sensing, natural transformation, oxidative stress, antibiotic resistance, motility, genome evolution, and hydrocarbon degradation [249]. These traits enhance their ability to thrive in wastewater environments, where biofilm formation and resistance to stressors are critical for survival and functional activity within the extreme environment of raw wastewaters [250]. *Moraxella* genus includes bacteria that are part of the human respiratory tract flora, and their presence in influent samples can be attributed to human-derived waste. Additionally, some *Moraxella* species are known

to thrive in nutrient-rich environments, which are characteristic of untreated wastewater [250,251]. Members of *Acidovorax* genus are commonly found in soil and water environments, including wastewaters, where it is one of the most abundant genera [252]. Their detection in influent samples suggests environmental runoff as a potential source, introducing these bacteria into the wastewater system. This runoff could result from agricultural activities, and industrial discharges, which carry soil-associated microbes and nutrients into the sewage.

On the other hand, the increased presence of species within the *Mycobacterium* genus in effluent samples may be due to their resilience to wastewater treatment processes. In fact, their cell wall structure provides resistance to disinfection methods, allowing them to persist through treatment and become more prominent in the effluents [253]. This genus is commonly found in WWTP environments [254]. *Zoogloea* genus is known for its role in forming activated sludge flocs, which are essential for the aggregation of bacteria during wastewater treatments [255,256]. The abundance of *Zoogloea* in effluent samples indicates its contribution to the flocculation process, enhancing the removal of organic matter and improving effluent quality. Although present in both influent and effluent samples, the reduced dominance of *Acidovorax* in effluents suggests that, while some species can survive treatment processes, their relative abundance decreases, possibly due to competition with other microbial populations better adapted to the treatment environment. In particular, *Acidovorax* has been commonly observed in WWTP that manifests a short sludge retention time [257]. Species of *Acidovorax* genus have been reported from WWTPs capable of aerobic heterotrophic growth and of anaerobic growth through denitrification [258,259].

These observations highlight the dynamic nature of microbial communities in wastewater treatment systems, influenced by factors such as nutrient availability, environmental conditions, and the inherent resilience of specific bacterial genera to treatment processes.

Effluent samples are accompanied by an increased presence of rarer genera, which suggests a higher diversification of the microbial community. These genera, typically present in low abundance, may play important roles in niche adaptation and ecosystem functioning, potentially reflecting changes in microbial interactions or resilience introduced by the treatment process. These changes not only reflect the effectiveness of microbial load reduction but also influence the ecological balance and functional potential of the effluent microbial community. Such a transformation is expected in properly operating wastewater treatment plant [260].

The diversity analysis showed that the effluent samples exhibited greater heterogeneity and higher taxa abundance (i.e., at family, genus, and species level) compared to the influent water samples. In

particular, Shannon entropy and Pielou's evenness indices indicate greater evenness in effluent samples, suggesting a more balanced community. Increased species diversity in the effluent waters is documented also in other studies [239,261,262]. This shift in microbial community composition in respect of influent waters is accompanied by an increase of rarer species, which can probably emerge because of the decrease of bacterial load in the water. This result could be linked to the survival of resilient microbial species or the elimination of dominant taxa, enabling rarer genera to thrive. Meanwhile, the removal of dominant taxa can reduce competitive exclusion, creating opportunities for less abundant organisms to establish ecological niches. These dynamics enhance the overall functional diversity and stability of the microbial community in the effluent. For instance, increased diversity may support nutrient cycling by reinforcing complementary metabolic pathways among different taxa. This suggests a significant ecological impact of the WWTP processes on microbial diversity, resulting in effluent waters hosting a microbial community more similar to that of the natural environment. This resemblance may be attributed to the reduced dominance of influent-specific genera and the proliferation of genera commonly found in natural aquatic ecosystems, such as *Mycobacterium* and *Zoogloea*. In addition, the increase in biodiversity during wastewater treatment may be related to the formation of biofilms in the WWTP environment [9,244]. However, given the numerous potential reasons for variations in microbial community structure, more comprehensive and in-depth studies are required to validate this hypothesis. It is important to consider that the increased representation of rare species in the effluent could be a result of diminished resolution during DNA extraction and sequencing in the influent samples. This reduction in resolution is possibly due to the overwhelming abundance of a few species in the influent, thereby impacting the detection of less abundant species.

A. johnsonii was the most prevalent in the influent waters, accounting for 18% of the total community. This could be attributed to several ecological and environmental factors:

- Metabolic versatility: *A. johnsonii* exhibits broad metabolic capacities, enabling it to thrive in diverse environments, including wastewaters. This versatility allows it to degrade complex organic molecules, such as hydrocarbons and aromatic compounds, which are abundant in untreated wastewaters. Furthermore, its ability to participate in nitrogen cycling processes, such as heterotrophic nitrification, supports its survival in nutrient-rich conditions characteristic of influent waters [249].
- Environmental reservoirs: this species is commonly found in natural environments, including soil and water bodies [263]. Its presence in wastewaters may reflect its ubiquity in the surrounding natural environment, leading to its introduction into WWTPs through surface runoff.

- Human activity: as an opportunistic pathogen, *A. johnsonii* is associated with human activities. Its prevalence in influent wastewaters could be linked to human waste, hospital effluents, or other anthropogenic sources, contributing to its elevated levels in wastewater systems [264].
- Resistance traits: studies have demonstrated that *Acinetobacter* species can harbor ARGs, which may confer a selective advantage in environments exposed to antimicrobial agents. The presence of such traits could facilitate the survival and proliferation of *A. johnsonii* in wastewater environments where antibiotic residues are present [265,266].

These factors collectively contribute to the dominance of *A. johnsonii* in influent wastewaters, underscoring its ecological adaptability and the dominance in the microbial community structures in influent wastewaters.

The ordination analysis via PCA, based on microbial community composition and abundance data from wastewater samples, shows that the three species that most significantly contribute to the differences among the samples are *A. temperans*, *M. osloensis*, and *A. johnsonii* (Figure 32). *A. temperans* is often associated with wastewater environments and plays a role in denitrification by reducing nitrate to nitrogen gas via enzymes such as nitrate reductase. Its ability to form biofilms and flocs enhances stability and efficiency within microbial communities, highlighting its adaptability to environments like WWTPs [267]. *M. osloensis*, known for its metabolic versatility, may indicate shifts in nutrient concentrations after the wastewater treatment, particularly through pathways such as aerobic denitrification and the degradation of complex organic compounds [268]. Consequently, *M. osloensis* could serve as a biomarker for effective nutrient removal and changes in wastewater treatment conditions. Its role in aerobic denitrification, for instance, might correlate with optimized nitrogen removal efficiencies under specific treatment regimes. These shifts often reflect variations in available carbon and nitrogen sources introduced during WWTPs treatment processes [269]. Notably, this species has demonstrated potential in enhancing nitrogen removal from wastewaters, particularly under low-temperature conditions. In fact, it maintained approximately 60% of its maximal growth activity at 10 °C and achieved significant removal efficiencies for ammonium, nitrate, and nitrite in wastewaters under low temperature conditions [268]. *A. johnsonii*, previously observed as a dominant species in the influent waters, reflects the selective pressures of the treatment process through its reduced presence in the effluent. These pressures likely include shifts in oxygen availability, competition for limited nutrients, and the selective removal of bacteria less suited to the operational conditions of the treatment system [270].

The uniformity and richness of the microbial community in the wastewaters changed significantly. Several studies have identified diverse microbial communities in the wastewater that could influence the microbial ecology of the connected ecosystem [271,272]. Understanding these dynamics can guide environmental management practices, such as mitigating the release of resistant or harmful microbes into natural water bodies.

4.2.2 Metagenome-free analysis: antibiotic resistance genes

WWTPs serve as critical sites for the concentration and dissemination of resistance genes in bacteria, thereby accelerating their spread into the environment [106,273]. Overall, the results of this study demonstrate that the treatment processes of the WWTP in Trieste efficiently reduced the abundance of ARGs prior to discharging the treated water into the sea, as reported in other studies worldwide [176,274–276]. In fact, despite the shared resistomes in both influent and effluent samples (Figure 35), the differential representation test (Table 18) showed a significant reduction in ARGs between the two sources. However, at the same time, this indicates the possibility of incomplete elimination of several pathogens carrying these genes in the WWTP.

Bacterial communities are considered to be one of the main determinants of ARGs distribution in wastewaters [277]. Previously observed changes in the microbial community in effluent waters resulted in a significant reduction in abundance of ARGs. This finding suggests a positive correlation between microbial biomass and absolute abundance of ARGs. The relationship might be due to the higher microbial densities providing more potential hosts for ARGs, thereby increasing their absolute abundance. Additionally, microbial biomass could influence the retention and persistence of ARGs during treatment processes, as denser microbial communities may preserve ARGs from degradation or removal [278]. This outcome was expected and aligns with previous findings where the reduction of ARGs in effluent waters was reported [31,139–141] and indicates that the treatment processes of the WWTP operate effectively and with moderate efficiency.

The treatment efficiency of the WWTP is highlighted in the PCA of the counts of ARGs, where strong clustering of samples by group (influent/effluent) was observed. This clustering shows clear distinctions in microbial community structure between influent and effluent waters, likely driven by the selective pressures exerted during the treatment process. The top four loadings, i.e., the ARGs with the greatest influence on this ordination, were identified as tet(Q), OXA beta-lactamase, ANT(3’’), and msrE. These genes, frequently detected in WWTPs, are functionally significant as they confer resistance to essential classes of antibiotics, including tetracyclines, beta-lactams, and macrolides. These antibiotics are considered essential due to their broad application in treating severe bacterial infections in both humans and animals. Resistance to these classes poses significant public

health risks by limiting the effectiveness of therapies, potentially leading to higher morbidity, mortality, and healthcare costs associated with AR infections [117]. Their presence underscores the adaptive capabilities of microbial communities in wastewater environments and highlights potential clinical risks if such genes are disseminated into natural ecosystems. In particular, tet(Q) encodes a ribosomal protection protein that confers resistance to tetracycline antibiotics. Studies have identified tet(Q) in various WWTPs, indicating its widespread presence in such environments [279]. It is found in both influent and effluent waters of WWTPs, indicating that conventional treatment processes may not fully eliminate this gene [280]. In the present study, the normalized abundance of tet(Q) shifts from 106 in the influent waters to 13 in the effluent ones.

OXA-type enzymes are class D β-lactamases capable of hydrolyzing β-lactam antibiotics, including penicillins and, in some cases, carbapenems. The bla_OXA genes have been detected in WWTPs, highlighting the role of these facilities as reservoirs and potential dissemination points for β-lactam resistance genes [155]. In this study, its concentration decreases from a normalized abundance value of 190 in influent samples to 23 in effluent samples (being the most abundant in effluents). ANT(3") gene encodes an aminoglycoside nucleotidyltransferase, which modifies aminoglycoside antibiotics, rendering them ineffective. It was reported that a subclass of aminoglycoside nucleotidyltransferase, ANT(3")-II, is widely distributed in the genus *Acinetobacter* [281]. It is relevant to note that, in the present study, this genus was the most prevalent in influent wastewaters, with *A. johnsonii* as one of the three species that most significantly contribute to the differences among the samples (as showed in the ordination analysis via PCA in Figure 32, based on microbial community composition and abundance data from wastewater samples). Also in this case the concentration of this ARG has notably decreased in the effluents (from a normalized abundance value of 122 to 13). The msrE gene encodes an ATP-binding cassette (ABC) transporter that confers resistance to macrolide antibiotics by actively effluxing them out of the bacterial cell [282]. Studies report the presence of msrE in wastewaters [283], commonly associated with *Acinetobacter* species [284]. This ARG is the most abundant in influent samples (normalized abundance value: 284) and it is present a low concentration in effluent samples (normalized abundance value: 16).

Overall, a significant reduction in ARGs quantities is observed, as the most abundant ARGs in the influent were 8~16-fold diminished in the effluent (Table 18). This reduction may be attributed to several factors, including the efficiency of physical processes such as sedimentation and filtration, the role of microbial degradation in removing ARG-carrying bacteria, and chemical treatments that target microbial cell walls or DNA. Additionally, operational parameters like hydraulic retention time and temperature likely influence the extent of ARGs removal.

These results reflect the efficiency of the WWTP in reducing the ARG levels, although their concentration is not completely reduced to zero. However, it is important to consider whether this level of removal is sufficient to mitigate environmental and public health risks. The residual ARGs in effluents may still contribute to the spread of resistance in downstream ecosystems (the effluent water of the WWTP is discharged into the sea), necessitating further evaluation of treatment processes and their long-term impact on resistance dissemination.

In addition, a total of 20 ARGs resistant towards different drug classes were abundant in the treated waters, and not in the raw wastewaters. Further analysis is necessary to elucidate and predict the possible factors influencing the presence of these unique ARGs in the resistome.

Moreover, some novel ARGs types in the CARD database, used here for identification and annotation, might have been overlooked due to the reliance of the RGI tool on similarity-based alignment, potentially leading to an underestimation of ARGs in samples.

4.2.3 Metagenome-free analysis: co-occurrence network analysis

Correlation networks analysis is one of the most commonly applied methods to visualize patterns in taxa or genes groups [285]. This method is particularly suitable for analyzing microbial communities and ARGs due to its ability to identify non-random associations, uncover underlying ecological interactions, and map the structural organization of complex datasets, which are pivotal for understanding microbial dynamics and resistance dissemination. This approach was employed to investigate the non-random co-occurrence patterns of genera-genera and ARGs-genera in the WWTP in Trieste to provide a deeper understanding of how ARGs and microbial community structures are organized within both influent and effluent water samples. Patterns of co-occurrence between nodes highlight distinct ecological niches, offering explanation for the favorable conditions of certain species toward enrichment and survival in the two sources [286].

Additionally, this analysis identified different module structure within the network, which reflect the influence of varying environmental conditions on population interactions and associations.

5 Influent samples

In the influent genera-ARGs network (Figure 36A), the presence of distinct clusters centered around particular ARGs hubs (most notably, VIM-7) suggests that certain ARGs may be key organizational nodes exerting influence over the genetic landscape of the community. The prominence of VIM-7, a well-known metallo-β-lactamase gene, points to strong associations with various other resistance determinants, possibly reflecting shared selective pressures, resistance mechanisms, or genetic

elements (e.g., plasmids, and integrons) that favor co-occurrence patterns in the incoming wastewater stream. The greater number of genera compared to ARG subtypes suggests that some bacteria may carry the same ARGs and act as hosts for specific ARGs. This observation highlights the potential for a shared resistance mechanism across diverse bacterial hosts, underscoring the importance of understanding these dynamics in predicting ARG dissemination within microbial communities. This is not necessarily attributed to HGT, the exact mechanism of which requires further research.

When the analysis was restricted to microbial genera alone (Figure 36B), the presence of multiple, independent clusters and moderate modularity (0.511) suggests that the community is relatively compartmentalized [287]. This compartmentalization may reflect the complexity of incoming wastewater sources (i.e., households, hospitals, industries) each potentially contributing distinct microbial and ARGs assemblages. The fact that ARGs removal subsequently increased the modularity in influent waters (from 0.41 to 0.51) indicates that ARGs may act as "connectors" bridging otherwise distinct microbial modules. This suggests that ARGs might facilitate interactions across microbial clusters, potentially influencing ecological stability and microbial diversity. In other words, the presence of ARGs within the network might introduce links that reduce the overall compartmentalization, possibly due to their association with mobile genetic elements that traverse taxonomic boundaries. On the other hand, it is worth underlining that the interactions between the genera communities (i.e., edges values) remain constant (1932 in influent, and 2000 in effluent). The key distinguishing factor is the presence of ARGs, as evidenced by the significant reduction in edge values from 13,184 in the influent to 5,141 in the effluent. This highlights the crucial role ARGs play in shaping the connectivity and organization of the bacterial community.

6 Effluent samples

In the effluent samples, the higher complexity of the co-occurrence networks aligns with the notion that biological treatment processes, such as activated sludge systems, promote dynamic interactions among diverse organisms and genetic elements [288]. The similarity in modular structure, i.e., three modules with central ARGs hubs between influent and effluent networks, suggests that some underlying organizational patterns persist throughout the treatment process. However, the increased complexity and the emergence of two distinct genera-based clusters, each with identifiable subclusters, demonstrate that the treated effluent environment may select for or enrich particular taxa. The identification of *Microthrix*, *Pararhodobacter*, and a yet-unknown genus of *Pseudonocardiaceae* as hub taxa within effluent communities is especially informative. These genera may be well-adapted to the conditions within the treatment system (e.g., nutrient availability, retention times, biofilm

formation), allowing them to occupy central ecological roles and potentially influence the community architecture and function.

Microthrix

Within the *Microthrix* genus, *Microthrix parvicella* is commonly found in activated sludge systems of WWTPs and is particularly well-known for its role in this environment as it can significantly impact the performance of biological processes. In fact, it is responsible for worldwide foaming and bulking problems in WWTPs with nutrient removal due to its hydrophobic cell surface. *M. parvicella* can uptake and store long-chain fatty acids under anaerobic conditions, subsequently metabolizing them under aerobic conditions. This unique physiological trait provides this species with a competitive advantage over most other activated-sludge bacteria in nutrient-removal plants that operate under dynamic anaerobic-aerobic conditions. Enhanced growth is also observed in wastewater with a high food-to-microorganism (F/M) ratio and in systems with low sludge loading rates [289,290].

Pararhodobacter

The genus *Pararhodobacter* belongs to the family *Rhodobacteraceae*, within the class *Alphaproteobacteria* and the order *Rhodobacterales*. It is a relatively recently described bacterial genus. It comprehends aerobic, chemoorganotrophic bacteria with strictly respiratory type of metabolism, which means that they rely on organic compounds for both energy and carbon. *P. aggregans* was isolated from a digestion basin, which was part of the biofilter of a recirculating marine aquaculture system in Rehovot, Israel [291]. *P. marinus sp.* was isolated from marine sediment sampled on Zhoushan Island located in the East China Sea [292]. Therefore, these bacteria are often found in marine or brackish water environments, playing roles in nitrogen and carbon cycling, particularly in aquatic ecosystems. This presence in the effluent waters of the WWTP can be linked to the intrinsic infrastructure of the plant, so called “which speaks with the sea” (“che parla con il mare”). WWTPs located near the sea often face unique challenges and dynamics due to the potential seepage or infiltration of seawater, especially during high tides or storm surges. In fact, rising sea levels and tidal fluctuations can increase hydrostatic pressure, pushing seawater into the wastewater network. This can lead to an increase of the salinity of the wastewaters and treated effluents, inhibiting biological treatment processes [293–297]. Thus, it may also alter the microbial community structure in selecting halotolerant or halophilic microorganisms [298,299].

Another hypothesis could be linked to the fact that halophilic bacteria represent a promising solution for managing saline wastewater, particularly in coastal and industrial regions. Their use in WWTPs

is of growing interest as saline wastewaters are challenging for non-halophilic microbes as their efficiency in biological treatment processes is reduced. Therefore, halophilic bacteria can survive in high-salinity conditions by maintaining metabolic activities, such as degrading organic pollutants, hydrocarbons, and certain recalcitrant compounds, where conventional bacteria fail [300,301].

Unknown genus of *Pseudonocardiaceae*

Members of the *Pseudonocardiaceae* family are aerobic, Gram-positive, non-motile bacteria that are commonly found in the environment. Their presence in effluent samples may indicate a potential role in wastewater treatment, particularly in the degradation of pollutants or resistance to certain environmental stressors, aligning with their known biotechnological applications. They have an important role in biotechnology due to the production of secondary metabolites like antibiotics, including erythromycin, rifamycin, and other polyketide-derived compounds (some of which have anti-bacterial, anti-fungal, and anti-tumour effects). Their ability to produce structurally diverse bioactive compounds makes them a focal point in antibiotic research, especially in the fight against multidrug-resistant pathogens. [302].

The observed increase in modularity after the removal of ARGs nodes in both the influent and effluent datasets implies that ARGs can blur natural community boundaries. By potentially moving horizontally among different microbial hosts and being enriched in certain parts of the treatment process, ARGs can integrate previously separate taxonomic clusters into more interconnected networks. Thus, the ARGs presence might be viewed as a factor that increases the overall connectivity and reduces the inherent “segmentation” of microbial communities. According to Faust and Raes, positive correlations between nodes of a network may imply co-occurrence, co-colonization, and/or co-aggregation [303]. It is important to note that this phenomenon could have been exacerbated by the limited number of replicates, potentially leading to an overestimation of correlations.

Collectively, these findings indicate that ARGs not only reflect but actively shape microbial community structure. The differences in network complexity between influent and effluent, alongside shifts in the role of ARGs, underscore the dynamic interplay between microbial communities and genetic elements over the course of the wastewater treatment. Future work will be needed to pinpoint the underlying genetic mechanisms, environmental pressures, and ecological interactions that drive these co-occurrence patterns. By doing so, a more comprehensive understanding of how ARGs

distributions and community structures evolve in engineered ecosystems may emerge, ultimately informing strategies aimed at mitigating the spread of antibiotic resistance.

Additionally, the co-occurrence network effectively highlights potential interactions between resistomes of interest and pathogenic hosts of concern. By extracting selected nodes from the global network and visualizing them separately, the associations between clusters in the meta-network can be more clearly illustrated. These findings provide a solid starting point for future research into the interactions between microbial populations and the mechanisms of gene transfer that contribute to the acquisition of AR in pathogens.

Furthermore, while the meta-network analysis was successfully performed, correlations between microbial taxa composition profiles and environmental factors such as temperature, pH, bioreactor pond depth, or the presence of metal particle pollutants were not explored. Such factors could significantly impact microbial dynamics and ARG distribution by shaping ecological niches, influencing microbial competition, or modifying resistance gene expression. Future studies could integrate these environmental variables to better understand their roles in structuring microbial communities and facilitating ARG dissemination.

The metagenomic analysis conducted in the WWTP of Trieste revealed the complexity of the microbial communities. It also provided a detailed functional profile of ARGs and identified patterns of co-occurrence between ARGs and their potential bacterial hosts.

6.1.1 Metagenome-based analysis

A limited number of studies have investigated the microbial community in WWTPs by comparing the influent and effluent waters using the shotgun metagenomic sequencing approach.

In the present study, the assembly metrics from multiple runs performed on the same sequencing data revealed differences between the two software tools compared in this study. Specifically, MEGAHIT consistently outperformed SPAdes, demonstrating superior assembly quality with a much lower computational footprint. Similar observations have been reported in other studies, where the performance gap becomes more pronounced with increasing sequencing depths [304–306]. This trend was confirmed in our analysis, where samples presented high sequencing depth and were sourced from a critical environment such as wastewater.

Most of the assembled contigs were small in size (<1 kb), introducing significant noise into the assembly process. Small contigs can complicate downstream analysis by reducing the accuracy of binning, as fragmented sequences may not be properly assigned to their originating genomes.

Additionally, they hinder functional annotation, as incomplete genes or pathways may not be identified, limiting the ability to characterize the metabolic and functional potential of the microbial community. This was particularly evident for the influent samples, where raw wastewater conditions contribute to challenges such as: nuclease activity, leading to degradation of nucleic acids; inhibitory substances, including chemical and biological inhibitors that interfere with nucleic acid extraction, amplification, and enzymatic reactions; low abundance of target genetic material, making accurate assembly difficult [307]. However, it is important to note that short contigs may also originate from plasmids, which are expected to be abundant in wastewater environments due to their role as mobile genetic elements. Plasmids are particularly relevant in this context as they often carry ARGs and virulence factors, facilitating HGT and contributing to the spread of resistance and pathogenicity within microbial communities [138].

After the removal of short sequences, the assembly metrics improved substantially, supporting the hypothesis that plasmid-derived sequences and fragmented assemblies were major contributors to the observed noise.

The analysis is ongoing, as downstream steps such as precise binning, taxonomic assignment, and functional annotation require substantial computational time and resources due to the vast amount of reference data involved. Nevertheless, preliminary outputs from CheckM suggest that several highly complete metagenome-assembled genomes (MAGs) were successfully reconstructed. This is a significant result, as it enables further annotation and functional analysis of the key microbial players in the wastewater environments, for both influent and effluent samples.

The large contigs that were binned together may allow the discovery of HGT and/or integration of ARGs between taxa and improve our understanding of these mechanisms in WWTPs.

From an ecological perspective, investigating metabolic pathways at the community level can reveal the strengths and weaknesses of the wastewater environment, providing insights into microbial metabolic potential and resilience. For example, pathways involved in nitrogen cycling, such as nitrification and denitrification, could highlight the role of specific taxa in removing nitrogenous compounds, while pathways for the degradation of complex organic pollutants can identify microbes contributing to the degradation of recalcitrant compounds. This information is critical for understanding ecological dynamics and optimizing treatment processes.

7 Conclusions

The removal of ARGs in wastewaters and the reduction of their spread in the environment remain critical areas of active research, given their implications for public health and ecological stability. WWTPs play a pivotal role in mitigating health risks associated with ARGs, particularly those stemming from waterborne infectious diseases and pathogen-contaminated water.

This metagenomics study contributes to addressing significant knowledge gaps regarding the microbial structure and ARG profiles in influent and effluent environments of the WWTP in Trieste (Italy), as well as the interactions between microbial species. The study provides solid evidence on the microbial community composition and ARG-bacterial genera associations, demonstrating that the core bacterial community observed aligns with previously reported microflora in conventional sewage treatment systems. While some microbial taxa are consistently detected in both influent and effluent samples, the overall microbial community composition changes substantially at lower biomass but higher biodiversity levels following treatment.

Despite these advancements, challenges persist in determining the precise roles of the identified microbial taxa within WWTPs, whether they act as key players or remain as minor contributors. Expanding analyses to identify plasmid-associated contigs and mobile genetic elements (MGEs) would offer deeper insights into the mechanisms of HGT, a critical vector for the dissemination of ARGs between microbial species. HGT not only facilitates microbial evolution but also significantly contributes to the spread of ARGs, leading to multidrug resistance in microorganisms.

To effectively control the rise and dissemination of antibiotic resistance, it is essential to formulate and implement targeted strategies. Increasing sampling locations and sequencing depth would enhance monitoring of microbial diversity and ARG dynamics over time. Future research should also explore correlations between wastewater strains and hospital clinical isolates to better understand the transmission pathways of ARGs and AR bacteria.

In conclusion, while significant progress has been made in understanding ARGs in wastewater systems, further research is required to identify functional roles of microbial taxa, improve detection of MGEs, and develop effective mitigation strategies. The ongoing application of metagenomics holds immense promise for addressing these challenges and advancing efforts to reduce ARG proliferation in wastewater and the environment.

The results obtained from this study could facilitate the assessment of antimicrobial resistance genes present in the bioaerosol by identifying specific antibiotic resistance genes, known to be present in the wastewaters, through the use of targeted PCR primers. This approach bypasses the need of a metagenomic analysis, which requires a large amount of genetic material, challenging to obtain from bioaerosol samples.

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Conclusions of the thesis

In the following paragraphs a recap is presented, resuming the main achievements of the thesis project, that aimed at the development and assessment of devices, procedures, and experimental settings for microbial bioaerosol characterization, and assessment of technologies of exposure mitigation and risk reduction.

The first two sections described the development – in laboratory setting – of original setups for the assessment of filtration devices (e.g., personal nasal filters, and a hyper-reflective UV-C LED aerodynamic filter module), that have been designed and assembled for the testing of the two typologies of filters, by aerosolization, transmission, sampling, and count of *E. coli* as model organism.

The further three sections move to indoor and outdoor applications of bioaerosol characterization in real world scenarios. The third section deals with the characterization of the spread of airborne culturable and settling bioaerosol through passive sampling and identification using 16S rRNA metabarcoding technique in one of the hospital wards renown for high bioaerosol production. This study focuses on a hospital ward known for high bioaerosol production, particularly due to mechanical procedures performed within the mouth of patients, namely the dentistry ward, with a case study conducted at the Maggiore Hospital in the city of Trieste (Italy). The fourth and fifth sections report studies at a type of technological plant where bioaerosols are formed constantly, that are of high interest for several aspects, among which the following can be cited: (1) aeration of untreated wastewaters represents a recognized source of bioaerosol – even if variable for composition of wastewaters and bioaerosol concentrations in proximity of water-air interface, depending on meteorological conditions; (2) wastewaters bring information about microorganisms and chemicals discharged “down the drain” by the whole community served by a sewer system, which can be also aerosolized at aeration tanks; (3) in wastewater treatment plants antimicrobial chemicals can exert selective pressure on microorganisms, promoting the survival of antimicrobial-resistant bacteria and the exchange of antimicrobial resistance genes, potentially leading to formation of new resistant species; (4) the very recent new EU wastewater directive – approved by the EU Council on November, 5th 2024 – that indicates the need of antimicrobial resistance monitoring. In particular, the fourth section focuses on an experimental comparison among different bioaerosol samplers, operating at very close position at the aeration tank of the wastewater treatment plant of Trieste (Italy), providing different results; the fifth section points at identifying broad spectrum antimicrobic resistant bacteria by high performing technologies as metagenomics (involving Next Generation Sequencing platform). The bioaerosol samplings did not yield sufficient genetic material for a metagenomic

study. Therefore, the focus has been directed to the characterization of antibiotic pressure, antimicrobial resistance genes and bacterial community in wastewaters that can be aerosolized. This approach aimed to define experimental and bioinformatics pipeline for characterizing the source prior aerosolization. In addition, the results of this study could facilitate the assessment of antimicrobial resistance genes present in the bioaerosol by identifying specific antibiotic resistance genes, known to be present in the wastewaters, using targeted PCR primers.

In the next lines specific results are summarized.

In the first section, a technology train and an experimental procedure for studying the behavior of bioaersol in controlled laboratory conditions has been designed and implemented, with the aim of testing microbial filtration performances of proposed innovative personal protection devices, as endonasal filters are. Our published design, tailored for assessing endonasal filter performance, evolved from our technology train constituted by a Blaustein Atomizing Module (BLAM) supported by an AERO Particle Nebulizer pump (TCR Tecora Srl), a custom cylindrical aerosol chamber, and a swirling bioaerosol collector connected to a Bio-Bravo sampling pump (TCR Tecora Srl). This setup was originally tested for evaluating residual infectivity after SARS-CoV-2 airborne transmission. The new design implemented a wider parallelepiped aerosol chamber (50 x 40 x 100 cm), potentially allowing *fine* bioaerosol accumulation for testing filters under critical conditions, and a support for the endonasal filters positioned within the aerosol chamber. This experimental setting attracted the attention of a private company, commissioning the Department of Chemical and Pharmaceutical Sciences of the University of Trieste to conduct preliminary testing of endonasal filters (with the results protected under a non-disclosure agreement, NDA). After experimentation with the aerosolization of non-pathogenic *E. coli*, the opportunity arose to upgrade the realism of tests by implementation of a sinusoidal breather (simulating human alternating inhalation and exhalation) instead of a constant flow pump; this would guarantee more turbulence within nasal filters, promote enhanced interaction of bioaerosol particles with internal filter walls, and potentially improve filtration performance. Improved experimental handling of bioaerosol in a laboratory setting and the capability of testing filtration technologies (personal protective equipment) operating at low flows (less than 20 L per minutes) have been achieved.

In the second section, the research systematically evaluated the performances of a UV-C LED aerodynamic device designed to inactivate airborne pathogens without incurring significant pressure losses. By adopting a methodical, data-driven strategy (interactions between analytical factors and their optimal levels were investigated by using sequential D-optimal designs adapted to domain constraints and previous computational simulations of the aerodynamic performance of the device), the study identified optimal power configurations, airflow parameters, and device geometries to

maximize bioaerosol inactivation, demonstrated using a nonpathogenic *E. coli* strain. This work underscored the pivotal role of airflow in pathogen inactivation by an UV-C LED and guided subsequent computational-aided design improvements. In fact, this experimental work was recognized as a driver for a computational-aided design of an improved bioaerosol inactivation device that has been published, performed by researchers from the Department of Engineering and Architecture of the University of Trieste, in collaboration with a spin off company (Esteco).

In the third section, a further study developed a procedure combining simple passive sampling and counts with the 16S rRNA metabarcoding technique to assess the presence of potentially hazardous bioaerosol generated during dental procedures, underscoring the risks posed to both healthcare workers and patients. This is particularly significant in the context of occupational health and infection control, where effective management of airborne microorganisms is critical to minimizing exposure to opportunistic pathogens and ensuring a safe clinical environment. Sampled colonies predominantly belong to the same genus *Staphylococcus*, which is noteworthy due to its relevance in both clinical and environmental contexts. This study also highlights an experimental strong correlation between dentistry activity and bioaerosol deposition rates - for what concerns relatively coarse bacterial bioareosol particles, prone to rapid settling. The high colony counts during operational hours reflect the generation of bioaerosols from dental procedures, while the decline during downtime suggests that culturable airborne bacterial particles settle over time when activities cease. These results underline the importance of targeted interventions in high-exposure zones (e.g., near the patient and lamp) to reduce local bioaerosol concentrations; this can be achieved e.g., by enhancing air exchange rates or by localized air filtration. Tailored advanced disinfection technologies, such as the one mentioned in Section 2, could be integrated into dental units. Research and development efforts are currently ongoing in this direction with the aim to capture bioaerosols generated near the patients prior to its dispersion in the indoor environment, thereby enhancing the overall air quality in dentistry clinics. Strategic placement of equipment – as high-contamination ultrasonic scalers - and barriers to segregate patient treatment zones can further limit bioaerosol spread. These strategies ensure that the proximity effect remains confined to safe levels and reduce the likelihood of cross-contamination within clinical spaces.

The fourth section addressed the lack of standardized sampling protocols for bioaerosol sampling by conducting an intercomparison study of multiple collection systems – filtration, swirling aerosol collection, and condensation growth tubes – near the aeration tank of the wastewater treatment plant of Trieste (Italy). Aiming at a broad characterization of bioaerosols, in absence of standardized approaches, the issue of selecting sampling devices able to guarantee adequate representativity of airborne microorganisms present in the air of interest assumes high relevance. An intercomparison

experiment has thus been conducted and published, considering parallel sampling by filtration, swirling aerosol collection, and condensation growth tube air sampling systems at a significant and continuous bioaerosol source, the aeration tank of the wastewater treatment plant. Collected samples were then analyzed using the 16S rRNA metabarcoding technique in order to identify bacterial taxa. In this screening comparison on bioaerosol samplers, a candidate device and collection medium has been identified for the bioaerosol sampling, suggesting that the BioSpot-VIVAS sampler with DNA/RNA Shield™ as collection medium is the best choice, despite lacking an optimal ease of operation due to its dimensions, weight, and need for operational parameter tuning. By taking into consideration smaller aerosol particles (i.e., <0.3 μm), the BioSpot-VIVAS sampler could allow to investigate the viral component of the bioaerosol as well. Experimental data – even if derived from a small number of samples - also highlight a difference in bacterial biodiversity between wastewater and air samples, with the latter being different depending on the device used based on the parameters set in this study (an overall sampling volume of 1440 L fixed for each sampler). The metabarcoding technique, combined with an automated sampling methodology, can be modified to validate and improve epidemiological models, as well as to organize early warning systems in biomonitoring approaches.

Finally, the fifth section extended the scope to include metagenomic profiling of wastewater microbial communities, focusing on bacterial biodiversity and antibiotic resistance. Due to limited genetic material obtained from bioaerosol samples, the study focused on the bacterial biodiversity of influent and effluent waters of the plant, with special attention on the presence of antimicrobial resistance genes (ARG), as well as the interactions between microbial species, contributing to address significant knowledge gaps. The study provides solid evidence on the microbial community composition and ARG-bacterial genera associations, demonstrating that the core bacterial community observed aligns with previously reported microflora in conventional sewage treatment systems. While some microbial taxa are consistently detected in both influent and effluent samples, the overall microbial community composition changes substantially at lower biomass but higher biodiversity levels following the plant treatment. Challenges still persist in determining the precise roles of the identified microbial taxa within wastewater treatment plants, whether they act as key players or remain as minor contributors. Expanding analyses to identify plasmid-associated contigs and mobile genetic elements would offer deeper insights into the mechanisms of horizontal gene transfer, a critical vector for the dissemination of ARGs between microbial species, leading to multidrug resistance in microorganisms. To effectively control the rise and dissemination of antibiotic resistance, it is essential to formulate and implement targeted strategies, as also foreseen by the new EU Wastewaters Directive. Future research should also explore correlations between wastewater

strains and hospital clinical isolates to better understand the transmission pathways of ARGs and antibiotic resistant bacteria. The ongoing application of metagenomics holds significant potential for addressing these challenges and advancing efforts to reduce ARGs proliferation in wastewaters and the broader environment.

In conclusion, this thesis integrates diverse methodologies to address a complex and underexplored topic, i.e., the bioaerosol. Key innovations include combining molecular and culture-based techniques and validating advanced air filtration systems. Section 1 reports the design of an experimental setting for the assessment of viability reduction of microorganisms and personal protection by biogel–silver nanoparticle endonasal filters: the study has attracted attention by a company, leading to experimentation that has produced results subject to a non-disclosure agreement. Section 2 describes experiments and optimization of parameters of a device developed from a patent partially owned by the University of Trieste for microorganism viability abatement; experimental results were considered by R&D of a spin-off company for improvement of the microorganism viability abatement module. Section 3 explored and characterized by both culture-based and molecular techniques bacterial spread in a hospital dentistry ward: results points at critical positions, and support – among risk mitigation strategies – the opportunity of localized air quality treatment, that can be achieved also by integration of microorganism viability abatement module in a dental unit. The development of a prototype of such technology is being developed in the frame of collaborations of the interdepartmental Bioaerosol and Air Quality Laboratory (BAQ Lab), involving a spin off company of the University of Trieste. Experimental results from Section 4 highlight the effectiveness of a relatively underutilized sampling technology, based on condensation growth tubes, for molecular metabarcoding characterization of bioaerosol bacterial biodiversity. This technology is distributed in Italy by XEarPro Srl, twin company of TCR-Tecora, which co-financing the doctoral grant. Aerosolization and sampling pumps deployed in instrumental trains developed in Sections 1 and 2 are produced by TCR-Tecora; a wet-cyclone prototype developed by TCR-Tecora has been tested in the WWTP as well, and critical points in the design have been identified (results not reported), supporting R&D units in product development. Lastly, the study presented in Section 5, which led to the development of an experimental and bioinformatic pipeline for the characterization of ARGs and bacteria in the WWTP, will open the way for the multi-utility company managing the plant to implement monitoring and treatment strategies for antimicrobial resistance, as foreseen by the EU wastewater Directive approved in recent November 2024.

The findings have broad implications for infection control, air quality management, and clinical safety. By providing actionable insights and practical solutions, this thesis significantly advances the field of bioaerosol research, setting the stage for future interdisciplinary studies.

Acknowledgements

I would like to express my gratitude to TCR-Tecora Srl for the financial support in co-financing my doctoral scholarship. A special acknowledgement goes to Dr. Maria Grazia Perrone for the unwavering collaboration, insightful discussions, and continuous exchange of ideas on the research and devices that have a central part of this thesis and beyond.

A heartfelt thank you goes to my supervisor, Prof. Pierluigi Barbieri, who taught me the importance of approaching science from an interdisciplinary perspective and conveyed to me the value of human relationships.
A very special thanks goes to Dr. Sabrina Semeraro, the lioness of our lab, who guided me through these three years both scientifically and personally, teaching me above all that “chi dorme non piglia pesci!”

I would also like to thank the entire team of the Applied and Comparative Genomics Lab in Building Q, starting with Prof. Alberto Pallavicini, Prof. Fiorella Florian, Dr. Fabrizia Gionechetti, Dr. Chiara Manfrin, ad Dr. Claudio Ametrano for all the scientific support and the affection they have shown me over the past five years. A big thank you also to Dr. Samuele Greco (another lion – who would have thought?) for his bioinformatics support and for being the desk mate with whom I could share all the silly things that crossed my mind (e.g., Shrek and SpongeBob memes). Thanks also to everyone else – Elettra, Beatrice, Carlotta, Sara, Marco, Azzurra, and Camilla – for sharing countless liters of coffee with me (and some scientific advice too!), accompanied by the scholarly readings of Paolo Fox’s horoscopes.

Returning to the Department of Chemistry (I know, stream-of-consciousness writing is just who I am, but I can’t help it – be grateful this isn’t a WhatsApp voice note), I would like to thank my research group: Dr. Enrico Greco, Prof. Sabina Licen, Dr. Stefano Fornasaro, and Dr. Felicia Sangermano for sharing these years with me.

I would like to thank those who welcomed me into the somewhat obscure world of chemistry.
First and foremost, my gratitude goes to the group made up of Demi, Marco, Michela, Rita, Carlo, Federico, Federica, Asja, and Chiara for sharing lunches, laughter, debates, and discussions (but we care about each other too!).
I would also like to thank those who first welcomed me into the department, with whom going out for an *aperitivo* is always a good choice: Giovanna, Miriam, Giuseppe, Smriti, Gaia (Steffy), Gaia (Chiara), Bogo, and Mario. Thank you for the shared lunches, UNO games, gossipz, and aperitifs. A special mention goes to Giovy for putting up with me as her roommate in Venice and Mestre (...lastly, just a brief mention about...!!).

I extend my heartfelt thanks to Alice Cespa for granting me the opportunity to conduct my research at the AcegasApsAmga wastewater treatment plant. My gratitude also goes to Giuseppe and Ivano, whose cheerfulness offset the less-than-ideal odors during the sampling days.

Lastly, thank you to all my friends outside the university, for whom I am immensely grateful and feel incredibly fortunate, as well as to my family and my cats.

Being surrounded by all these people has made all the difference ☺