Sequencing Batch Biofilter Granular Reactor (SBBGR) for wastewater treatment and irrigation reuse

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Sequencing Batch Biofilter Granular Reactor (SBBGR) for wastewater treatment and irrigation reuse

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EXTENDED ABSTRACT *(eng)*

In the last decades, both world governmental organizations and public opinion have become aware of water as a precious and limited resource. The increase in world population and climate change suggest the need for a more rational use of water, the reuse of treated wastewater turns out to be a promising strategy to preserve water resources and increase the availability of water in different sectors.

The technologies used for wastewater treatment are based primarily on activated sludge systems. These technologies have several limitations such as the need for large treatment surfaces, low biomass settling properties and high excess sludge production, but above all, they do not guarantee sufficient pathogens removal in case of reuse of treated wastewater. Conventional wastewater treatments require a process consisting of several purification steps and need a disinfection phase to obtain microbiologically safe effluent aimed to irrigation. These disadvantages have prompted the scientific community to study new treatment systems able to guarantee the quality of treated water and the minimum environmental impact, in order to more easily meet water demand in agricultural sector.

Among the new systems recently proposed that can comply with this request, the SBBGR system, developed by the Water Research Institute (IRSA) of the Italian National Research Council (CNR), seems to be a promising technology. The aim of this study is the evaluation of SBBGR as a system able to increases the simplification of the treatment scheme for treating and reusing municipal raw wastewater and improves the management of water demand and supply.

In the present study, a larger group of physical, chemical and microbiological parameters was considered for evaluating the effectiveness of the SBBGR system for municipal wastewater treatment and agricultural reuse. SBBGR enhancement with sand filtration and chemical (by peracetic acid, PAA) or physical (by UV radiation) disinfection was also evaluated to have a high effluent quality needed for irrigation.

The monitored parameters were chosen according to the current Italian regulation and their representativeness. In particular, the microbial indicators were chosen considering that most human pathogens that could be derived from the reuse of wastewater belong to the domains of bacteria, viruses and protozoa, and these microorganisms are characterized by different physiological characteristics and consequently different survival rates in

wastewater treatment. Furthermore the spreading of antibiotic resistent genes (ARGs) into the environment has recently raised a great concern for the risk of wastewater reuse as vehicle for genes, for this the removal SBBGR capability of four genes (ermB, sul1, sul2, tetA) resistant to different classes of antibiotics was evaluated in effluent after biological treatment and after tertiary phase.

The SBBGR resulted really efficient in removing suspended solids, COD, BOD and nitrogen with an average effluent concentration of 4, 40, 1 and 14 mg/L, respectively. Lower removal efficiency was observed for phosphorus with an average concentration in the effluent of 3.7 mg/L. Plant effluent was also characterized by an average electrical conductivity and sodium adsorption ratio of 0.88 mS/cm and 3.8, respectively. Therefore, according to these gross parameters, the SBBGR effluent was conformed to the national standards required in Italy for agricultural reuse. Moreover, disinfection performances of the SBBGR was higher than that of conventional municipal wastewater treatment plants and met the quality criteria suggested by WHO (*Escherichia coli* <1000 CFU/100 mL) for agricultural reuse. In particular, the biological treatment by SBBGR removed, 2.5 ± 0.8 log units of total coliforms, 3.2 ± 1.1 log units of *E. coli*, 1.1 ± 0.4 log units of *Clostridium perfringens,* 1.4 ± 0.3 log units of Somatic coliphages, 1.5 ± 0.9 log units of Giardia and 1.8 ± 0.3 log units of Cryptosporidium. The investigated disinfection processes (UV and peracetic acid) resulted very effective for total coliforms, *E. coli* and somatic coliphages. In particular, a UV radiation and peracetic acid doses of 40 mJ/cm^2 and 1 mg/L respectively reduced *E. coli* content in the effluent below the limit for agricultural reuse in Italy (10 CFU/100 mL). Conversely, they were both ineffective on *C. perfringens* spores.

The SBBGR system is able to reduce 1-2 logarithmic units of ARGs. In particular, a removal of 1.6 ± 0.7 log units was recorded for *ermB*, 1.9 ± 0.8 LUR for *sul1* and 2.2 ± 1.1 for *tetA*, while for *sul2* there was a lower decrease compared to the other genes, 1.0 ± 0.4. No reduction of the ARGs amount normalized to the total bacteria content (16S rDNA) was obtained, indicating that these genes are removed together with total bacteria and not specifically eliminated. Enhanced ARGs removal was obtained by sand filtration, while no reduction was achieved by both UV and PAA disinfection treatments tested.

key word: Wastewater treatment, SBBGR, Water reuse, Pathogens, *E.coli*, Antibiotic resistance genes.

EXTENDED ABSTRACT *(ita)*

Negli ultimi decenni sia le organizzazioni governative mondiali sia l'opinione pubblica hanno acquisito consapevolezza riguardo l'acqua come risorsa preziosa e limitata.
L'aumento della popolazione mondiale e i cambiamenti climatici suggeriscono la necessità di un uso più razionale delle risorse idriche, infatti il riuso delle acque reflue trattate risulta essere una strategia promettente per preservare le risorse idriche e incrementare la disponibilità di acqua in diversi settori.
Le tecnologie impiegate per la depurazione delle acque di scarico sono basate principalmente su sistemi a fanghi attivi, ma questi presentano alcuni limiti come la necessità di ampie superfici per il trattamento, bassa velocità di sedimentazione della biomassa e alta produzione di fanghi di supero, ma soprattutto, non garantiscono una rimozione sufficiente degli agenti patogeni in caso di riutilizzo delle acque reflue trattate. I trattamenti convenzionali sono caratterizzati da un processo composto da diverse fasi di purificazione e richiedono uno stadio di disinfezione per ottenere un effluente microbiologicamente sicuro destinato all'irrigazione. Questi svantaggi hanno spinto la comunità scientifica a studiare nuovi sistemi di trattamento in grado di garantire la qualità dell'acqua trattata e il minimo impatto ambientale, in modo da poter più facilmente rispondere alla richiesta d'acqua del settore agricolo.
Tra i nuovi sistemi recentemente proposti che possono soddisfare questa richiesta, il sistema SBBGR (Sequencing Batch Biofilter Granular Reactor), sviluppato nell'ultimo decennio dall'Istituto di Ricerca Sulle Acque (IRSA) del Consiglio Nazionale delle Ricerche (CNR), sembra essere una tecnologia promettente. Lo scopo di questo studio è la valutazione della tecnologia SBBGR, come un sistema in grado di aumentare la semplificazione dello schema di trattamento per il riutilizzo delle acque reflue urbane grezze e migliorare così la gestione della risorsa idrica.
In questo lavoro sono stati indagati diversi parametri chimici, fisici e microbiologici per valutare l'efficacia del sistema SBBGR nel trattamento delle acque reflue destinate al riuso agricolo. Inoltre è stato valutato il sistema SBBGR potenziato con filtro a sabbia e con una fase di disinfezione chimica (tramite utilizzo di acido peracetico PAA) o fisica (tramite utilizzo di raggi UV) per soddisfare i criteri di qualità richiesti in caso di riuso per irrigazione.

I parametri monitorati sono stati scelti in base all'attuale normativa Italiana e alla loro rappresentatività. In particolare, gli indicatori microbici sono stati scelti considerando che la maggior parte dei patogeni umani che potrebbero derivare dal riutilizzo delle acque reflue appartengono a batteri, virus e protozoi e questi microrganismi sono caratterizzati da diverse caratteristiche fisiologiche e conseguentemente diversi tassi di sopravvivenza durante il trattamento delle acque reflue. Inoltre la diffusione di geni resistenti agli antibiotici (ARGs) nell'ambiente ha recentemente sollevato una grande preoccupazione per il rischio di riutilizzo delle acque reflue, individuate come veicolo per questi geni. Per questo motivo è stata valutata, nell'effluente dopo il trattamento biologico e dopo la fase terziaria, la capacità di rimozione della linea di trattamento nei confronti di quattro geni resistenti a diverse classi di antibiotici.

Il sistema SBBGR è risultato efficace nella rimozione di solidi sospesi, COD, BOD e azoto con una concentrazione media nell'effluente rispettivamente pari a 4, 40, 1 e 14 mg/L. Per il fosforo è stata osservata una minore efficienza di rimozione con una concentrazione media nell'effluente di 3.7 mg/L. L'effluente era inoltre caratterizzato da una conducibilità elettrica media di 0.88 mS/cm e un rapporto di adsorbimento di sodio di 3.8. Pertanto, in base a questi parametri, l'effluente SBBGR è risultato conforme agli standard nazionali richiesti in Italia per il riutilizzo agricolo. Inoltre, le performances di disinfezione del sistema SBBGR sono apparse superiori a quelle degli impianti convenzionali di trattamento delle acque reflue municipali e in linea con i criteri di qualità suggeriti dall'OMS (*Escherichia coli* <1000 CFU/100 mL) per il riutilizzo agricolo. In particolare, il trattamento biologico di SBBGR ha rimosso, 2.5 ± 0.8 unità logaritmiche di coliformi totali, 3.2 ± 1.1 unità logaritmiche di *E. coli*, 1.1 ± 0.4 unità logaritmiche di *Clostridium perfringens*, 1.4 ± 0.3 unità logaritmiche di coliformi somatici, 1.5 ± 0.9 unità di *Giardia* e 1.8 ± 0.3 unità logaritmiche di *Cryptosporidium*. I processi di disinfezione studiati (UV e acido peracetico) sono risultati molto efficaci per i coliformi totali, *E. coli* e colifagi somatici. In particolare, la radiazione UV di 40 mJ/cm^2 e 1 mg/L di acido peracetico hanno ridotto il contenuto di *E. coli* nell'effluente a valori inferiori al limite per il riutilizzo agricolo in Italia (10 CFU/100 mL). Al contrario entrambi sono risultati inefficaci sulle spore di *C. perfringens*.

Il sistema SBBGR è in grado di ridurre 1-2 unità logaritmiche di ARG. In particolare, è stata registrata una rimozione di 1.6 ± 0.7 unità logaritmiche per *ermB*, 1.9 ± 0.8 per *sul1* e 2.2 ± 1.1 per *tetA*, mentre per *sul2* c'è stata una diminuzione inferiore rispetto agli altri

geni (1 ± 0.4). Non è stata ottenuta riduzione della quantità di ARG normalizzata al contenuto totale di batteri (16S rDNA), indicando che questi geni sono stati rimossi insieme con i batteri e non specificatamente eliminati. Maggiore rimozione degli ARG è stata ottenuta mediante filtrazione a sabbia, mentre nessuna riduzione è stata ottenuta con i trattamenti di disinfezione, sia UV che PAA.

key words: Trattamento acque reflue, SBBGR, Riuso acqua, Patogeni*, E.coli,* Geni antibiotico-resistenti.

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1. INTRODUCTION

United Nations Department of Economic and Social Affairs has foreseen a growth of the world population of 7.7 billion people in 2017 to one between 9.4 and 10.2 billion in 2050. Population growth, economic development and new consumption patterns will cause a growing increase in water use. Global water withdrawals will continue to grow and there will be increasingly strong imbalances between water supply and demand. Starting from 2010, around 1.9 billion people (27% of the world population) live in areas with potential severe water scarcity. Many countries are already facing the problem of water scarcity and in a few decades they will have a decline in water resources that will worsen their condition and create new risk areas (Fig.1.1.). Domestic use of water should grow significantly mainly due to of the adaptation of water supply and sewerage services in the expanding urban settlements and the construction of new services in countries with developing or emerging economies. A strong increase in water demand is also expected for industrial production, responsible for the 20% of water extraction in the world and above all for the agricultural sector, 70% of available water is used for irrigation. Water withdrawals for irrigation purposes are now recognized as a primary factor in reducing the quantities of water worldwide. Since 2010, has been registered an increase in the use of freshwater, mainly for agricultural use, of 800 km^3 a year (Burek et al., 2016). According to estimates, if the "business-as-usual" conditions are maintained, to meet the expected increase of the demand for food products a larger area of cultivable land and a greater quantity of water to irrigate them will be needed. Starting from 2050 a considerable increase in water withdrawals is expected, up to 1,100 km^3 per year, an increase of 39% compared to current levels (Burek et al., 2016).

According to this, reuse ot treated wastewater in agriculture could provide an effective alternative to meet agriculture's demand and also increase freshwater resources for other needs. One of the major barriers to water reclamation and reuse is concerns regarding the health risk of exposing public to treated wastewater and associated contaminants (Zhang et al. 2007). Despite the high interest in wastewater reuse in agriculture, common quality standards for this aim are still missing at European level (Salgot and Folch 2018). In fact, some countries have limited quality criteria to a few main parameters (i.e. COD, suspended solids, total coliforms, faecal coliforms or *E. coli*), while other countries have

adopted more stringent parameters, including emerging pollutants and different kinds of microbial indicators (Brissaud, 2006; Salgot et al. 2006; Li et al. 2009). To achieve the safety standards issued by the different regulations, conventional wastewater treatments require several purification steps in order to obtain effluents with a quality suitable for re-use in agriculture.

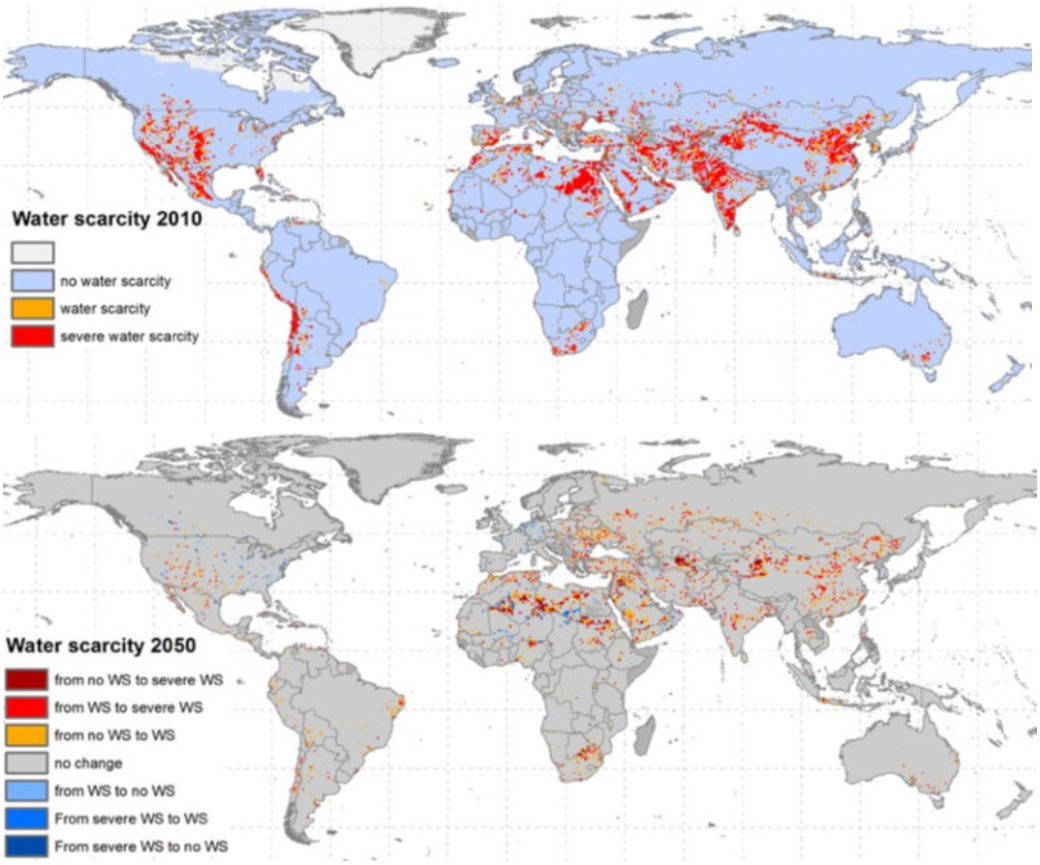

Fig.1.1. Water scarcity in 2010 and expected changes in water scarcity by 2050 (UNESCO 2018).

To facilitate treated wastewater reuse in agriculture it is necessary to design simplified treatment plants. With proper management and treatment processes, wastewater could represent a new resource for agricultural needs, in the context of a more rational use of water.

2.1. WASTEWATER

2.1.1. Urban Wastewater characterization

Social, productive and recreational activities require and use a large amount of water. Contaminants, impurities and pollutants are added to the water during its use, so water lose the original quality characteristics and becomes wastewater. Water quality is an essential element for the correct management of water resource, for its use and for its treatment and disposal in receiving water bodies, respecting the environment. Wastewater originates predominantly from water usage by residences, commercial and industrial establishments, together with storm water. These waters, whose quality has been impaired, are channeled into sewage networks as discharges. Sewage is a complex mixture of inorganic and organic substances that must be treated appropriately before final disposal. Urban wastewater has extremely variable characteristics, which depend on several factors, including the number of inhabitants served, the per capita water supply in addition to the degree of industrialization. The characteristics of urban wastewater, therefore, depend not only on human metabolic activity, which however represents the greatest contribution, but also on the fact that in domestic practice we use chemical products such as detergents, solvents, oils and acids. It is also necessary to consider the contribution of drainage waters in urban areas, such as roads, squares, etc., and the presence in urban centers of utilities such as laundries, service stations, artisanal workshops and garages that discharge industrial effluents into sewers. Untreated wastewater generally contains high levels of organic material, numerous pathogenic microorganisms, solids as well as nutrients and toxic compounds.

Wastewater is classified as strong, medium or weak, depending on its contaminant concentration. The low, medium and high load conditions are related to the per capita water supply respectively 750, 460 and 240 liters/inhabitant · day. (Table 2.1.1.)

An understanding of the nature of wastewater is fundamental for the design of appropriate wastewater treatment plants and the selection of effective treatment technologies. Wastewater quality may be defined by its physical, chemical, and biological characteristics.

Contaminants	Unit	Concentration		
		Weak	Medium	Strong
Total solids (TS)	mg/L	350	720	1 200
Total dissolved solids (TDS)	mg/L	250	500	850
Fixed	mg/L	145	300	525
Volatile	mg/L	105	200	325
Suspended solids	mg/L	100	220	350
Fixed	mg/L	20	55	75
Volatile	mg/L	80	165	275
Settleable solids	mL/L	5	10	20
BOD_5, 20°C	mg/L	110	220	400
TOC	mg/L	80	160	290
COD	mg/L	250	500	1 000
Nitrogen (total as N)	mg/L	20	40	85
Organic	mg/L	8	15	35
Free ammonia	mg/L	12	25	50
Nitrites	mg/L	0	0	0
Nitrates	mg/L	0	0	0
Phosphorus (total as P)	mg/L	4	8	15
Organic	mg/L	1	3	5
Inorganic	mg/L	3	5	10
Chlorides	mg/L	30	50	100
Sulfate	mg/L	20	30	50
Alkalinity (as $CaCO_3$)	mg/L	50	100	200
Grease	mg/L	50	100	150
Total coliforms	No/100 ml	10^6-10^7	10^7-10^8	10^7-10^9
Volatile organic compounds	µg/L	<100	100-400	>400

Tab. 2.1.1 Typical composition of untreated domestic wastewater (Metcalf & Eddy, 2006).

2.1.1.1. Physical parameters

Physical parameters include colour, odour, temperature and turbidity. Insoluble contents such as solids, oil and grease, also fall into this category. These parameters identify the basic properties of wastewater and are the first level of characterization.

Temperature

The wastewater temperature is normally higher than that of tap water due to domestic and industrial hot water discharges, and it depends on geographical location and season. The importance of wastewater temperature is fundamental both in terms of the treatment process and of environmental impact.

The oxygen solubility is a function of temperature, therefore the aerobic processes are directly influenced, moreover, the temperature affects the speed of microorganisms biochemical reactions. The discharge of water at a temperature different than that of the receiving water body can have severe effects on the indigenous fauna and flora.

Color

The color of the wastewater is closely related to its septicity, fresh sewage with time becomes dark due to the natural decay of organic matter, while an abnormal coloring of the waste indicates the strong presence of industrial discharges. Colored wastewater introduced into the environment can alter the natural transparency of water bodies and affect photosynthesis reactions.

Odour

The foul-smelling emissions are essentially linked to the gaseous compounds produced in the decomposition of organic and sulphurated substances. The odorigenous compounds present in already treated wastewater, indicate a low efficiency of the treatment.

Solids

If untreated wastewater is discharged to the aquatic environment, solids can lead to the development of sludge deposits, anaerobic conditions and alterations in ecosystem balance. The term "solids" in a liquid means all those substances that it is possible to detect when evaporation of the liquid has occurred.

Total solids (TS) in wastewater consist of the insoluble or suspended solids, colloidal material and the soluble compound dissolved in water, constitute the residue of the drying of a sample of wastewater placed at a temperature of 105 ± 5° C. Total solids are classified into:

• Total dissolved solids (TDS) are those that are not retained by a porosity filter of 0.45 µm after drying at 105 ± 5 °C. They include colloidal solids and dissolved solids (i.e. dissolved salts) which influence conductivity.

• Total suspended solids (TSS), portion of total solids retained by a porosity filter of 0.45 µm after drying at 105 ± 5 °C. Sedimentable solids represent a fraction of suspended solids, have a density higher than that of the liquid, are calculated with an Imhoff cone, ex-

pressed as volume (ml/l), they are an approximate measure of the amount of mud that will be removed from the primary sedimentation and represent about 60% of the total suspended solids.
TSSs are further divided into:
- Volatile suspended solids (VSS), a portion that volatilizes after burning the TSS at a temperature of 600 ° C;
- Fixed suspended solids (FSS), residual portion after burning the TSS at 600 ° C.
The VSS represent an estimate of the organic matter in the solids, while the fixed represent the inorganic matter.

2.1.1.2. Chemical parameters

The chemical characterization of wastewater requires a more complex analytical investigation aimed at identifying and quantifying the main substances present in the discharges.

Organic matter
The organic substances found in wastewater are proteins (40 to 60%), carbohydrates (25 to 50%), fats and oils (8-12%) and fractions of other compounds. Urea is another important compound present in the wastewater (main constituent of urine). However, due to its rapid decomposition into ammonia inside the sewage system, urea is only found in fresh wastewater.
Chemical parameters associated with the organic content of wastewater include chemical oxygen demand (COD) and biochemical oxygen demand (BOD).
The measurement of BOD allows to estimate the concentration of biodegradable organic substances, this parameter is the measurement of dissolved oxygen used by microorganisms in the biochemical oxidation of the organic substance. The most used parameter as an indicator of organic pollution is the 5-day BOD (BOD_5), after an incubation period of 5 days at 20 °C (being the biological phenomena strongly influenced by temperature). The higher is the amount of biodegradable substance, the greater will be the amount of oxygen required by microorganisms to degrade it.

The COD test also estimates the biorefractory component. It, measure the equivalent amount of oxygen needed to chemically oxidize the organic matter present in the sample. The COD of a sewage is, in general, higher than the BOD because by the chemical way it is possible to oxidize also biorefractory (non-biodegradable) pollutants and because in the biological process (used for the BOD measurement) a fraction of the pollutant substrate is used in the anabolic phase of cellular metabolism (i.e. to generate new biomass).
The BOD_5/COD ratio is indicative of the biological treatability of a wastewater, in particular:
values between 0.5 and 0.6 indicate that the waste is highly biodegradable;
values between 0.2 and 0.3 indicate that the wastewater is highly recalcitrant.

Nitrogen compound

Nitrogen and phosphorus are essentialy the most important nutrients for microorganisms' growth in biological wastewater treatment. In the raw sewage nitrogens occurs in the form of ammonia (which is found as NH_4^+ or as NH_3 depending on the pH) and of organic compounds such as aminoacids, nitrogen sugars and proteins. The organic nitrogen undergoes processes of transformation and degradation during the time spent in the sewage network and is converted into ammonia nitrogen, substrate of nitrification bacterial process. Ammonia nitrogen undergoes a process of nitrification and possibly even denitrification, during the treatment of waste water to avoid the negative impact on the aquatic environments. Nitrite and nitrate are respectively intermediate and final products of the ammonia oxidation reaction. Nitrite (nitrous nitrogen) is relatively unstable and easily oxidized to nitrates or reduced to gaseous nitrogen. The study of nitrite pollution is very important because of their extreme toxicity to fish and other aquatic species. The concentration of nitrates (nitric nitrogen) increases as nitrification proceeds. The removal of nitrates from discharges is strictly necessary to avoid eutrophication.
The determinations of organic nitrogen and ammonia are based on the Kjeldahl method, the sum of TKN, nitrites and nitrates (the two oxidized forms) constitutes the total nitrogen, indicated with TN (total nitrogen).

Phosphorus

Phosphorus in wastewater derives from sanitary waste (40%) and phosphates present in household detergents (60%), it is present in three different forms: orthophosphate, polyphosphate and organic phosphate. Phosphorus compounds are indispensable for biomass growth in biological treatment systems, but must be removed before re-entry into the environment. One of the main effects of phosphorus on water is eutrophication. Like nitrogen, also an excessive presence of phosphates soluble in water gives rise to an abnormal fertilizing effect for the aquatic vegetation. The end result is a disproportionate growth of algae and therefore a decrease of dissolved oxygen.

pH

The concentration of hydrogen ions is one of the fundamental parameters of control in biological processes. The microorganisms used in water treatment processes are compatible with limited pH values, in the range between 6 and 8, therefore wastewater with unfavorable pH is difficult to treat biologically. Biological oxidation processes tend to reduce the pH, alkalinity is an indicator of the buffer capacity of the medium (resistance to variation in pH) due to the presence of bicarbonate, carbonate and hydroxyl ions.

Salinity

The salts dissolved in the water determine conductivity value, the highest is the value of conductivity, the greater the amount of mineral salts dissolved in the water. Inorganic chemical parameters include also hardness,and alkalinity but they are searched only in specific cases.

Metals

Traces of metals such as nickel (Ni), manganese (Mn), lead (Pb), chromium (Cr), cadmium (Cd), zinc (Zn), copper (Cu), iron (Fe) and mercury (Hg), are commonly present in waters. Although many of these metals are classified as pollutants, much is needed, in very low concentrations (a few mg/L) for biological growth, and their absence can act as a limiting factor. Their concentration is monitored especially in the presence of industrial discharges.

2.1.1.3. Microbiological parameters

For complete qualitative water characterization it is necessary to know, beside the physico-chemical features, also the biological characteristics. In wastewater there are a large number of microorganisms including bacteria, fungi, algae, protozoa, plant and animal species and even viruses. In particular pathogenic microorganisms deriving from human or animal infected dejections deserve particular attention. Among the pathogens we identify four major categories: bacteria, protozoa, helminths and viruses.

The most common pathogens present in the wastewater belong to the Salmonella species, and are able to cause diseases commonly known as salmonellosis in humans. The most severe form of infection is typhoid fever caused by *Salmonella typhy*. A less common bacteria, *Shigella*, is responsible for intestinal diseases known as bacillary dysentery or shigellosis. In the waste waters, bacteria belonging to the Vibrio, Mycobacterium, Clostridium, Leptospira, and Yersinia species have also been isolated. Several cases of water-borne gastroenteritis are reported, whose etiologic agents are thought to be some normally non-pathogenic gram-negative bacteria, such as *Escherichia coli* and some Pseudomonas species. (Crook, 1998). Protozoa, unicellular eukaryotic microorganisms such as *Cryptosporidium*, *Cyclospora* and *Giardia* are present in almost all wastewaters. These micro-organisms are of high interest because of their strong impact on individuals with immune deficiencies, including very young people, the elderly, cancer and AIDS patients.

Both reoviruses and adenoviruses have been isolated in the wastewater, whose effects in terms of respiratory diseases, gastroenteritis and eye infections are known.

Humans are able to produce more than 100 different types of enteroviruses that are responsible for infections and diseases. Enteroviruses multiply in the intestinal tract of infected individuals and are released from them through the faeces.

Another important biological parameter is the presence in wastewater of bacteria and genes antibiotic-resistant, ARBs and ARGs respectively. The extensive and sometimes unappropriated use of antibiotics for both human health and animal husbandry, has led to the emergence of antibiotic resistance. Indeed, some bacteria are resistant to the action of antimicrobial drugs. The ARGs are usually carried by mobile genetic elements (plasmids,transposons, integrons) and can multiply in their hosts as well as, be transferred to

other bacteria and be subjected to further evolution (Lupo et al., 2012).The release of these microorganisms into the environment, through urban wastewater, could contribute to the spread of antibiotic resistance.

2.1.2. Estimate of the flow rates

Sewage networks are designed to capture, collect and transfer the sewage produced by human activity to a treatment plant where the fluids will be subjected to purification processes before being reintegrated into the natural water cycle. In the case of residential areas, the volumes of wastewater produced are estimated on the basis of the resident population and the per capita water supply representing the consumption of water related to each individual inhabitant expressed as $L \cdot inhabitant^{-1} \cdot day^{-1}$
In the absence of rainfall, the flow rate of a civil waste can be assessed as a fraction of the flow distributed by the supply system (equation 1):

$$(q_{24})_c = (\alpha_c \cdot DI \cdot P)/1000 \qquad \text{(Eq. 1)}$$

$(q_{24})_c$ = annual average flow produced by a civil user, m^3/d
α_c = coefficient of flow into sewage, considered equal to 0.8
DI = per capita water supply, l/ab/d
P = resident population, inhabitants
1/1000 = conversion factor from liters (*DI*) to m^3 ($(q_{24})_c$) (De Feo et al. 2012)

Usually, an average flow into the sewer is considered equal to 80% of water consumption. The volumetric flow rates of wastewater depend on the quantitative and qualitative characteristics of the water supply, but also on the level of water services offered. The unit flow rates for commercial buildings can vary considerably, so a careful analysis must be carried out for different structures of commercial activity and on the basis of the number of employees. Furthermore wastewater flow rates for many recreational facilities are subject to seasonal variations which must be analyzed and considered for the design of the plant.

2.1.3. Regulatory issues

The "Merli Law", No. 319/1976 "Rules for the protection of water from pollution", represented the first italian regulatory reference on water pollution. This legislation mainly regulates the limits of chemical substances present in industrial and civil discharges but did not directly regulate the discharges of municipal treatment plants. The limits were established in order to protect the species *salmo irideus Gairdneri*, fish chosen as a indicator for the protection of water bodies. Subsequently with the legislative decree 152/1999 is regulated the quality of the municipal discharges and the quality of the receptors water bodies. This decree imposes more stringent limits for parameters like BOD, COD, solids, total nitrogen and phosphorus for all treated civil wastewater and suggests to carry toxicity test using *Daphnia magna* species.

Various modifications and additions have led to the issue of Consolidated Environmental Act (Legislative Decree No. 152/2006). The rules of this decree constitute the transposition and implementation of Directive 96/61/EC on integrated pollution prevention and reduction, and Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for action in the field of water. In Decree 152/2006 there are rules relating to air, waste, contaminated sites and in particular in Part Three "Rules on soil protection and combating desertification, protecting water from pollution and managing water resources" there are regulations concerning water. Legislative Decree 152/2006 defines urban wastewater as "the mixture of domestic wastewater, industrial wastewater and run-off meteorites, channeled into sewage systems, even separate, and coming from urban agglomeration" (urban agglomeration: “areas in which the population and productive activities are concentrated in such a way as to render admissible, technically and economically, the conveyance of urban waste water to one treatment system or to a final drop-point”). The limits for discharges are differentiated according to the type of receptor water body and to the potentiality of the treatment plant, measured in number of population equivalent (PE) for each PE it is considered a biodegradable organic load having value of BOD_5 equal to 60 gr O_2 per day.

Furthermore the decree establishes the technical rules for the reuse of domestic, urban and industrial wastewater through the regulation of the intended use and the related quality requirements, for the purpose of the qualitative and quantitative protection of water re-

sources. In particular, it indicates three possibility of re-use of these recovered waters: in the agricultural field for irrigation, in the civil field for street washing, for the supply of heating and cooling systems, in the industrial field for the availability of fire-fighting water or for washing.

In addition to Legislative Decree 152/06, Ministerial Decree 185 dated 12 June 2003, which establishes the technical regulations for the reuse of domestic waste water, similar to domestic, urban and industrial waste, is also in force, indicating the limits for the different chemical and biological parameters within which waters values must be included for reuse in the various fields.

For wastewater coming from domestic settlements, among the parameters to be taken into consideration and which must fall within the tabular limits imposed by Ministerial Decree 185/2003 (ANNEX 1), there are the following: BOD_5 - *Biochemical Oxigen Demand*, it is the biochemical demand for oxygen from the bacteria present in the treatment systems, necessary for the biodegradation of the organic substance. The limit imposed for this parameter by the D.M. 185/03 is 20 mg O_2/L; COD - *Chemical Oxigen Demand*, is the chemical oxygen demand necessary for the chemical oxidation of substances present in domestic wastewater. The limit imposed for this parameter by the D.M. 185/03 is 100 mg O_2/L; TSS -Total Suspended Solids - They can be coarse solids, sediment solids and non-sedimental solids, and depending on their characteristics, they may require different purification treatment systems, which in domestic wastewater are mainly of physical nature, without the use of products chemical. The limit imposed for this parameter by the D.M. 185/03 is 10 mg/L; NITROGEN and PHOSPHORUS - high concentrations of nitrogen and phosphorus in wastewater must always be avoided, as eutrophication phenomena of surface water courses of discharge receptors can develop, causing anomalous development of microalgae and bacteria, which causes the death of fish due to lack of dissolved oxygen. The risk of eutrophication is high only if nitrogen and phosphorus are both present in high quantities.The limits imposed for these parameters by the D.M. 185/03 is 2 mg P/L for Phosphorus and 15 mg N/L for total Nitrogen. In agriculture, these two elements, if well dosed, are instead necessary to fertilize the crops and therefore the local authorities are delegated by the D.M. to increase these values to 10 mg P/L for Phosphorus and to 35 mg N/L for total Nitrogen, without prejudice to the provisions of art. 10, paragraph 1, concerning the areas vulnerable to nitrates of agricultural origin. It is evident, in

fact, that it is simpler and less expensive not to eliminate them from the effluent, rather than eliminate and then reintegrate them.

Regarding microbiological parameters, *Escherichia coli* and *Salmonella* are considered. The legislative decree 185/03 foresees for the reuse of the treated waters a maximum concentration of 10MPN/100mL of *E.coli* and the absence of *Salmonella*. Also the *Giardia* and *Cryptosporidium* protozoa, parasites rarely researched in the Italian waters, have recently been indicated by the WHO as emerging pathogens with water diffusion. The detection of microorganisms in irrigation waters does not involve problems of plant phytotoxicity or problems with the soil, but regarding the hygienic-sanitary aspect of the product obtained.

From 12 December 2017 the European Law 20 November 2017, n. 167 "Provisions for the fulfillment of obligations deriving from Italy's membership of the European Union - European Law 2017" came into force. In particular, Article 16 integrates the rules dictated by Legislative Decree 152/2006 relating to the methods of analysis used for monitoring the status of water bodies; Article 17 refers to the emission limits for urban wastewater plants delivering to sensitive areas and Article 18 modifies some rules concerning industrial emissions.

2.2. URBAN WASTEWATER TREATMENT

2.2.1. Conventional treatment of urban wastewater

Municipal wastewater treatment plants (WWTPs) typically consist of preliminary treatment, primary treatment and secondary treatment. A higher degree of treatment, defined as tertiary, is necessary to improve the quality of the effluent in order to meet the limits established according to the final destination of the treated wastewater. Disinfection technologies is used to further reduce pathogen microrganisms below the standards set out for hygienic-sanitary safety in case of reuse. (figure 2.2.1).

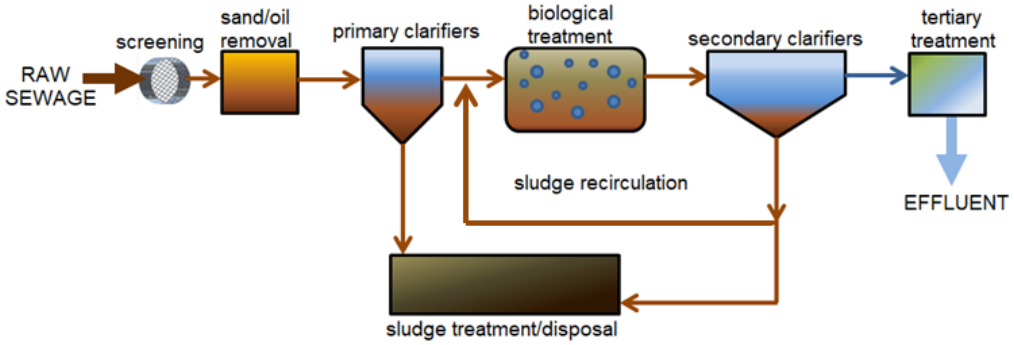

Fig 2.2.1 Conventional treatment scheme.

2.2.1.1. Preliminary and primary treatment

The first methods of treatment used are based on physical operations, in which physical forces are applied to remove the contaminants. The preliminary treatments, in addition to removing a fraction of the sewage pollution, constitute a guarantee to safeguard the correct functioning of the subsequent treatments; in fact the gross materials and the sand, which could damage the mechanical equipment of the plant and slow down the whole process, are removed.

Screening: a grid retains solid and voluminous materials.The grids are positioned inside a chamber or a channel, inclined to the flow of raw sewage; the quantity and quality of re-

tained material depends on the size between the bars of the grid and on the characteristics of the slurry. The principal types of screening devices are listed in table 2.2.1.

Screen category	Size of openings (millimeters)	Application	Types of sceens
Coarse screens	30-60	Remove large solids, rags, and debris.	• Manually cleaned bar screens/trash racks • Mechanically cleaned bar screens/trash racks - Chain or cable driven with front or back cleaning - Reciprocating rake screens - Catenary screens - Continuous self-cleaning screens
Fine screens	15-20	Reduce suspended solids to primary treatment levels.	• Rotary-drum screens • Rotary-drum screens with outward or inward flow • Rotary-vertical-disk screens • Inclined revolving disc screens • Traveling water screens • Endless band screen • Vibrating screens
Microscreens	0.2-2	Reduce suspended solids to primary treatment levels	

Tab. 2.2.1. Screen types. (Adapted from Liu and Lipták,2000).

Then a sand removal is carried out, to remove soil, sand, gravel and crushed stone, possibly present because washed away during rains. De-oiling is also carried out, through which oils, greases and other substances with a density lower than water are removed. The latter is realized by blowing minute bubbles of air from the bottom of a tank to cause flotation on the surface of fats and other materials, which are then removed by a mechanical spill.

For optimal operation, a wastewater with a constant flow rate and homogeneous polluting loads would be appropriate. Therefore, WWTPs usually include a unit for equalization and homogenization of the wastewater, equalization is the operation to regulate the flow rates while homogenization regulates the polluting load.
Primary settlement tanks are generally circular in shape and ending in cone so that the heaviest sludge is collected at the bottom. There are tow main designs, namely longitudinal flow sedimenters, with fluid movement from the inlet to the outlet end, and radial flow sedimenters, moving from the center to the border. The suspended solids that gradually settle constitute the so-called primary sludge. It is important that the system must produce both a clarified effluent and a concentrated sludge, the efficiency of the primary settlement tank is dependent on the type of solids present in the wastewater and the retention time of the wastewater in the tank. The heaviest solid organic materials held in suspension by the turbulence of the water in a quiet state are deposited; the lighter and more floating materials come to the surface. Sludge is removed regularly from the bottom of the tank through a scraping device and conveied to an outlet duct, while a shallow blade removes the floating materials.

2.2.1.2. Secondary treatment

Secondary treatments also known as biological treatments are based on the consumption of organic matter, present in both particulate and dissolved form and nutrients by microorganisms. Biological processes exploit the ability of some microbial communities to remove organic matter, while producing secondary sludge (or biological sludge). The microorganisms commonly used in secondary treatments are aerobic. They oxidize the organic and inorganic substance present in the wastewater, using oxygen as the final acceptor of electrons, in order to produce energy to reproduce (production of new microorganisms) and to support their vital functions. Most heterotrophic organisms can directly oxidize organic carbon to carbon dioxide (catabolic phase of cellular metabolism), using oxygen or nitrites and nitrates (in this case is a denitrification process) as electron acceptors. The energy produced by the catabolic phase is used in the cellular synthesis process, in which part of the organic carbon present in the wastewater is used to produce the

cellular components of the new microorganisms (anabolic phase of the cellular metabolism).

The whole process can be summarized as follows:

$$\underset{\text{organic matter}}{COHNS} + O_2 + \text{nutrients} \xrightarrow{\text{bacteria}} CO_2 + NH_3 + \underset{\text{new cell}}{C_5H_7O_2N} + \text{other end-products}$$

In addition to the oxidation of organic matter, microorganisms may also be able to remove nitrogen, through the nitrification and denitrification process. The nitrification consists in the oxidation of ammonia first to nitrite and then to nitrate :

Nitrosation:

$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H+ + 2H_2O$

Nitration:

$2NO_2^- + O_2 \rightarrow 2NO_3^-$

Nitrification:

$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$

The first conversion is carried out mainly by the bacteria belonging to the genus Nitrosomonas and Nitrosospira, while the second from the genera Nitrospira and Nitrobacter. Nitrifying bacteria are chemo-lithotrophic organisms, able to derive energy from the oxidation of inorganic compounds. Moreover, they are autotrophic, as they use carbon dioxide as a carbon source, and are therefore characterized by a low growth rate.

The nitrification process is influenced markedly by the pH value (values below 6.5 lead to the stopping of the process), by the temperature (low temperatures slow down the nitrification kinetics) and by the concentration of dissolved oxygen (the nitrifying bacteria are competing for oxygen with microorganisms that oxidize the organic substance).

The final stage of the nitrogen removal process consists of denitrification, which takes place under anoxic conditions (absence of dissolved oxygen). It consists in the reduction of nitrites or nitrates to gaseous nitrogen (N_2) with simultaneus oxidation of the organic substance, by heterotrophic bacteria (the same ones that in the presence of oxygen oxi-

dize the organic substance). There are many kinds of bacteria able to perform anoxic respiration: among these the most common in treatment plants belong to the genera *Pseudomonas*, *Micrococcus*, *Bacillus*, *Spirillum*, *Thauera* and *Azoarcus*.

2.2.1.2.1. Activated sludge process

The activated sludge (AS) process was developed in 1914 by Arden and Lockett. It was so called because it involved the produciion of an activated mass of microorganisms capable of aerobically stabilising the organic content of a waste. Nowadays the AS process is the most used biological process for the treatment of civil waste.
In the biological treatment with activated sludge a controlled dynamic aerobic system is created in the tanks, reproducing in the artificial environment the same biological mechanisms that occur in nature but with faster reactions and in a smaller space. The active biomass is kept in suspension through suitable mixing and aeration systems in order to guarantee the continuous mixing with the slurry and the right amount of oxygen necessary for biological oxidation. The characteristic of AS systems is the nature of the sludge, in fact there are several bacterial populations that coexist and work in the process: heterotrophs, nitrifiers, denitrifiers, polyphosphate and glycogen accumulating organisms. The microbial flora used to treat wastewater, instead of remaining dispersed in the treated effluent, tends to aggregate forming flocs of variable size between 50-500 μm. These floccose particles if placed in quiet conditions (secondary clarifiers), tend to sediment and can be separated from the clarified sewage that remains on the surface (supernatant). The sedimented biomass, named activated sludge due to the presence of active microorganisms, is recycled into the aeration tank, while a fraction is periodically removed, because it is excess of the treatment requirements, and constitutes the excess sludge of secondary treatments (Metcalf & Eddy, 2006).
The main limit of activated sludg sistem is due to the light flocs, that can not reach high concentrations inside the reaction tanks. These systems are characterized by low purification capacity and, therefore, to guarantee the necessary treatment capacity, they require high reaction volumes which are obtained by increasing the surface area (an action which is often hampered by the unavailability of land near the plant), with negative impli-

cations as regards both the production of smells, noises and aerosols, and the transfer of oxygen.

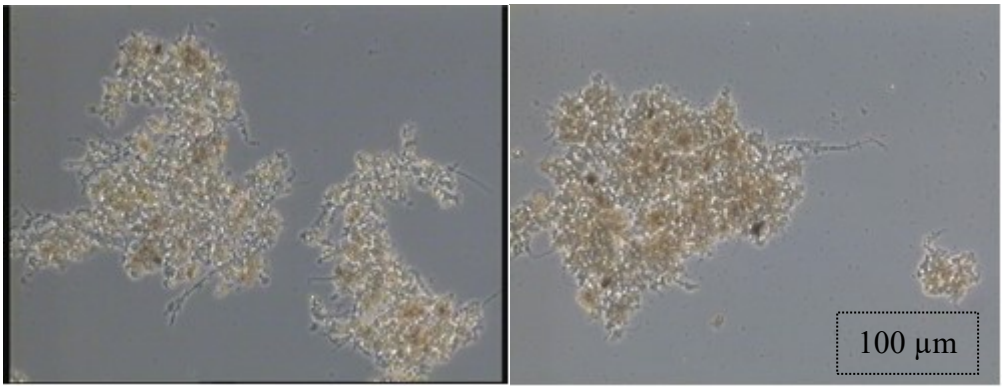

Fig. 2.2.2 Phase contrast photo of activated sludge flocs (100x magnification).

Also the separation of the biomass from the purified effluent, due to low sedimentation speed of flocs, requires units (sedimenters) of considerable size, which often are not available. Furthermore, the sedimentation capacity can be compromised due to alterations in the sludge structure. Some toxic substances can cause problems in bioflocculation leading to a biomass characterized by single dispersed cells. The structure of the flocs also depends on the presence of filamentous bacteria that compete with the flocs forming microorganisms; poor presence of filamentous bacteria leads to the formation of pin point flocs of very small dimensions, instead the excessive growth of filamentous bacteria compromises the ability of the flakes to separate.

Well settling activated sludge should have the following properties (Wanner, 1994):

- it settles with zone settling velocities of >3 m/h,
- it does not occupy an excessive volume after settling and thickening in a secondary clarifier,
- after sedimentationin leaves a clear supernatant,
- it does not rise after sedimentation for at least 2-3 h.

When activated sludge does not fulfil these features, it escape from the secondary clarifier and deteriorates final effluent quality not only in terms of suspended solids but also in BOD_5, COD, TN and TP (due to the biomass content of N and P) (Rossetti et al. 2017).

The main residue of the treatment process is the sludge produced during both primary and secondary sedimentation. The sludge is conveyed into the sludge line to be appropriately treated and disposed of, representing one of the major plant management problems both from a technical and a ecomnomic point of view. Unfortunately, activated sludge systems are characterized by a high production of sludge, whose disposal in the environment is increasingly problematic and constitutes a rise in the operating costs of the entire plant.

2.2.1.3. Tertiary treatments

Tertiary treatment goes beyond the level of conventional secondary treatment to remove biologically non-degradable refractory component, heavy metals, microorganisms and other pollutants (United Nations 2003). Different reuse applications require different water quality specifications and thus demand different treatments (Li et al. 2009). Furthermore the final treatment presents disinfection step when water is destined to a use that requires a particular hygienic-sanitary protection. The need to disinfect wastewater, in particular urban wastewater, stems from the fact that they can be vehicles of faecal pathogens.
Primary and secondary treatments are generally not sufficient to reduce the pathogenic charge to the required levels, especially if WWTP effluent is reused for crops irrigation (Li et al. 2009; Akhoundi and Nazif, 2018; Intriago et al. 2018). Depending on the potential of the purification plant, it may therefore be necessary to disinfect the secondary effluent by different technologies also combined. The quality of the secondary effluent affects not only the kind of technology to be used, but above all the dosage of disinfectant needed to reach the quality levels required.
Some reclamation technologies are used for preparing the water to be easly disinfected, fore example the filtration of effluents from secondary treatment processes is widely used for the supplemental removal of suspended solids and dissolved unbiodegradable organic compounds (Salgot and Folchn. 2018). The complete filtration operation comprises two phases: filtration and cleaning or backwashing.
The wastewater to be filtered is passed through a filter bed consisting of granular material (sand, anthracite and/or garnet), with or without added chemicals. Within the filter bed, suspended solids contained in the wastewater are removed by means of a complex process involving one or more removal mechanisms such as straining, interception, impac-

tion, sedimentation, flocculation and adsorption. The cleaning/backwashing phase differs, depending on whether the filter operation is continuous or semicontinuous. In semi-continuous filtration, the filtering and cleaning operations occur sequentially, whereas in continuous filtration the filtering and cleaning operations occur simultaneously (Metcalf & Eddy, 2006).

The assessment of the microbiological quality of water is carried out through the research of indicator microorganisms. With regard to the control of emissions of water discharges, the legislation identifies the *E. coli* parameter as the primary indicator of faecal contamination. Therefore, the main purpose of disinfection processes is to achieve a significant reduction in the abundance of this microorganism in the purified effluent.

Disinfection is usually carried out with a chemical reagents, usually chlorine (chlorination), even if in recent years they are increasing more and more the applications that see the use of ozone, peracetic acid and physical agents (ultraviolet rays) (Bonetta et al. 2017, Collivignarelli et al. 2017)

Chlorine is an oxidizing agent which is in the form of hypochlorous acid and hypochlorite ion. Chlorine enters the cell and given its reactivity with the nitrogen compounds, denatures especially the enzymes. Hypochlorous acid diffuses into the cell more rapidly than hypochlorite. Unfortunately, the toxicity of some products that are formed during the process (for example, chloramines) is limiting this application (de Souza et al., 2015)

Peracetic acid (PAA) is a carboxylic peracid produced by the reaction of hydrogen peroxide (HP) with acetic acid (AA). PAA is a strong disinfectant and oxidant (its oxidation potential is higher than that of chlorine). The pure product is extremely reactive and unstable, in fact the PAA is commercially available in the form of an equilibrium mixture containing AA, HP, PAA, and water (Kitis 2004). The PAA disinfection mechanism is due to the release of 'active' oxygen, which in turn destroys the sulfhydric bonds (-SH) and disulfides (-SS-) (Gehr 2003). With this oxidative destruction, PAA causes the breaking of important components inside the cell membranes; crossing the cell membranes of microorganisms attacks the enzymatic system and is thus able to inhibit their vital processes. There is no release of toxic by-products, in fact the decomposition of the peracetic acid in water leads to the formation of oxygen, water and acetic acid which at most can lead to an increase in BOD and COD in the final effluent (Collivignarelli et al., 2017).

Ozone (O_3) destroys micro-organisms by acting as a protoplasmic oxidant. Reacts with water to give free radicals that are strong oxidants. Ozone, however, is very unstable, can not be stored or transported, so it must be produced directly on site with a generator (commonly called ozonator). It has a low solubility in water and therefore requires special engineering measures in order to obtain a high transfer yield.

The term 'ultraviolet rays' refers to those electromagnetic radiations which cover the wavelength range from 100 to 400 nm and are divided into UV-C (from 200 to 280 nm), UV-B (from 280 to 320 nm) and UV-A (from 320 to 400 nm). The germicidal action of UV rays is due to photochemical alterations. DNA and RNA are the most important light energy absorbers in the UV-C wavelength range and being carriers of genetic information for reproduction, their alteration damages the cells by deactivating their vital functions. UV rays provide energy to the DNA, bringing some of its parts into a state of excitement and inducing alterations. They trigger a reaction that binds two thymes present on the double helix (Figure 2.2.3.), this coupling called dimerization prevents an accurate transcription of the DNA strand and the bacterial cell can not divide (Gehr 2003).

To obtain a high disinfection efficiency with UV rays, an effluent practically free of suspended solids is required. The presence of solids in the water to be treated is the main limit to the application of UV rays, since bacterial colonies can lodge in the suspended solids and protect themselves from radiation; the dissolved solids (inorganic and organic substances), moreover, reduce the transmittance of the water and therefore the intensity of the radiation.

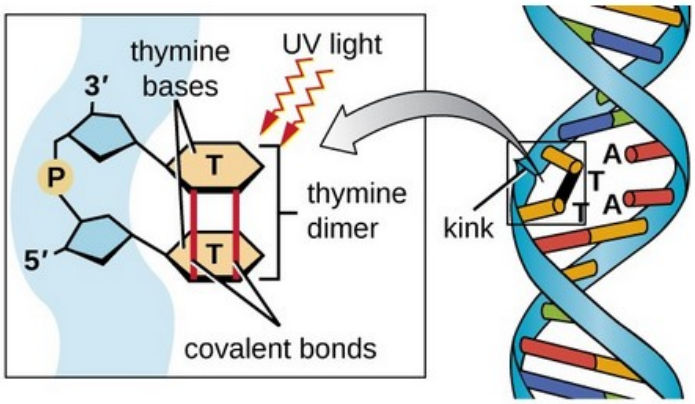

Fig 2.2.3. Damage to DNA by UV rays (courses.lumenlearning.com).

Ultraviolet rays have the advantage of not producing toxic by-products; the effluent disinfected with UV rays, however, shows phenomena of "regrowth" of the microbial population under certain conditions with time (Koivunen and Heinonen-Tanski 2005.)
The fundamental element of the UV reactor is the radiation source, consisting of mercury vapor lamps. Lamps consist of quartz tubes or similar materials (UV-permeable) filled with an inert gas and small amounts of mercury vapor, and are equipped with two electrodes: the passage of the current between the two electrodes determines the formation of an arc electric, which induces the excitation of mercury atoms that emit radiation. The intensity of the radiation emitted at the various wavelengths depends on the partial pressure of mercury in the lamp. The low pressure lamps operate with partial pressures of Hg <1 bar (between 0.01 and 0.1 bar), temperatures around 40-50 °C and power supplies of 10-350 W/lamp. They are protected by a quartz sheath, sometimes Teflon, which allows UV rays to pass without great absorption. The protection also has the function of avoiding excessive drops in temperature, if the lamp is put in direct contact with the liquid, so as to maintain as much as possible the optimal temperature, between 35 and 50 °C. These lamps convert 35-40% of the electrical energy absorbed into light radiation and in practice are monochromatic, with 85% of the radiation having a wavelength of 254 nm of maximum efficiency. They have a life span of 4000 to 15000 hours, even if after about a year their performance decreases by 30-40%, so it is still recommended to replace them. They are particularly suitable for small systems, even if there are no shortage of applications on medium systems, for which the application of a large number of lamps becomes necessary. The medium pressure lamps are so called because they operate with partial pressures of Hg>1 bar (in practice between 1 and 6 bar). They work with much higher temperatures (600-900 °C), therefore there are problems of heat dissipation. In this case, the share of light energy in the wavelength range of 254 nm is much lower (15-20%) and even shorter the duration of the lamps (3000-4000 hours). Their only advantage, often however decisive, is that they can operate with very high powers (from 1 to 30 kW) and therefore they find specific use on large plants. There are also high pressure lamps (6-10 bar), however in the experimental phase.

2.2.2 Alternative system for wastewater treatment

As previously described, conventional activated sludge systems require large surfaces and have some limitations such as sludge settleability, washout from clarifiers, production of excess sludge. Major concern of water authorities are: minimising cost of wastewater treatment; meeting effluent requirements; area availability (de Kreuk at al. 2007). To meet these needs in recent years new treatment systems have been studied.

2.2.2.1. Membrane Biological Reactor

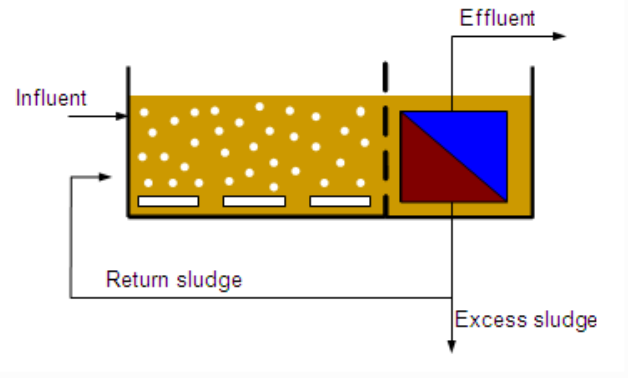

Fig 2.2.4. Membrane Biological Reactor treatment scheme (emis.vito.be).

MBR (Membrane Biological Reactor) systems are based on the combination of active sludge processes with filtration technology (figure 2.2.4.) The biological process for removal of organic matter and nitrification is comparable with that of activated sludge systems, but MBR systems allow a better biomass separation without the secondary sedimentation phase (Judd et al. 2008). MBR treatment processes are not affected by sludge settling problems and this makes it possible to achieve 3-4 times more biomass concentrations compared to activated sludge systems. Membranes work as selective barriers with microscopic pores, the passage of the effluent through the membranes allows to retain solids, bacteria and viruses. The use of membranes allows to obtain greater efficien-

cy in removing contaminants with the possibility of reusing the effluent (Melin et al. 2006). Despite an excellent quality of the effluent, the MBR system has disadvantages mainly related to management and economic aspects. The membranes are quite expensive and are subject to deterioration and fouling of the pores. In addition there is a high energy consumption due to both the filtration operations and to the washing and maintenance of the membranes.

2.2.2.2. Moving Bad BioReactor

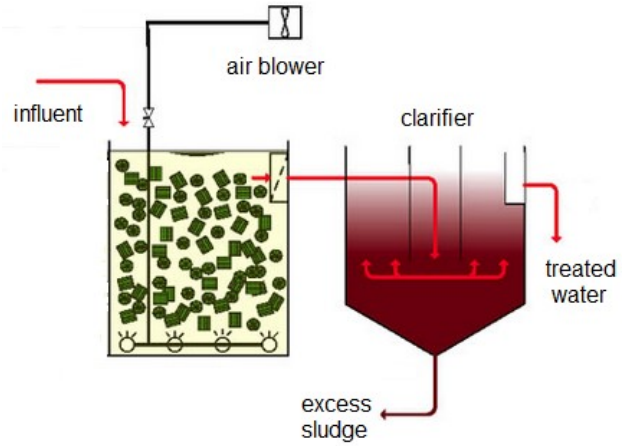

Fig 2.2.5. Moving Bad BioReactor treatment scheme (www.enviropro.co.uk)

MBBR (mobile bed reactors) are biological reactors similar to activated sludge reactors but integrated with the presence of a porous support (usually plastic material) medium dispersed in the biological tank (figure 2.2.5.). Thanks to this system, in addition to free biomass, there is the growth of biomass attached to the plastic material. This feature makes it possible to increase the concentration of biomass in the system resulting in a smaller footprint compared to a conventional activated sludge process (Ødegaard 2006). The particular conditions of the biological tank allow nitrification and denitrification processes thanks to the formation of aerobic and anoxic zones inside the biofilm. Further advantages are represented by the greater resistance to load variations and by the flexibility of the system, which allows adaptation to changes in organic load by changing the filling rate of the biological tank. In the treatment scheme it is necessary the presence of nets at

the exit of the biological tank to prevent the loss of the biocarriers and furthermore, a secondary settler is needed to separate the free biomass and the excess biofilm, which detaches from the carriers, from the effluent. The process is configured differently based on the patents of the filling material (Captor®, Linpor® or Kaldnes®). The supports differ in shape, material size, density, porosity and specific surface; their movement in the biological tank is guaranteed by aeration in aerobic systems and by mechanical stirrer in anoxic/anaerobic systems.

2.2.2.3. Sequencing Batch Reactor

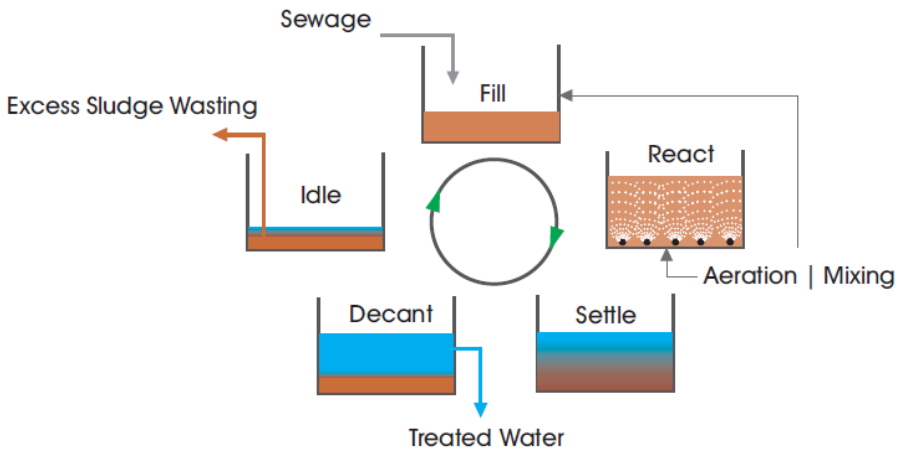

Fig 2.2.6. Sequencing Batch Reactor treatment scheme (oceanidesglobal.com).

SBR (Sequencing Batch Reactor) systems are based on a time-oriented operation process characterized by a discontinuous feeding phase (fiigure 2.2.6.) The various phases that make up the biological process are then performed cyclically in time sequence in the same reactor (Irvine et al. 1989). These systems consist of a single unit in which the biological oxidation and sedimentation processes are developed and from which the extraction of both the purified effluent and the excess sludge is carried out.This separation over time of the process steps allows a reduction in the size of the plants, external settling tanks are not needed. Furthermore in SBR systems there is a lower incidence of the filamentous bulking phenomenon. The operating cycle can be modified to meet effluent lim-

its and accomodate fluctuation in flow rate, but if the supply wastewater exceeds the capacity of the SBR a buffer tank is needed for the influent.

2.2.2.4 Granular sludge reactors

The study of the transformation of flocculent biomass into stable aggregates started from the mid-seventies. Granular sludge aggregates are particular stratifications of microorganisms showing significantly different morphological and structural characteristics than activated sludge flocs. The special properties of granules have encouraged the study of new treatment processes. Granulation was first observed in anaerobic systems known with the acronym of UASB (Upflow Anaerobic Sludge Blanket) (Lettinga et all.,1980).
Systems with anaerobic granular sludge allow high loading rates, require low energy for mixing, but also have several disadvantages: the anaerobic systems are characterized by good efficiency in removing the organic substance but have minimal removal of nitrogen and phosphorus, require high operating temperatures and long start-up periods due to the slow growth rate of microorganisms.
The granulation process has been applied since the 1990's also to aerobic systems, using reactors operating in discontinuous called GSBR reactors (Granular Sequencing Batch Reactors) (Morgenroth et al., 1997). Granulation is the process by which the self-immobilization of microorganisms leads to the formation of dense agglomerates. Liu and Tay (2004) defined aerobic granules as highly packaged microbial aggregates, containing millions of microorganisms per gram of biomass, in which the different bacterial species have specific functions in the degradation of pollutants present in the wastewater.
Due to the oxygen penetration gradient, there are different layers in the same granule. According to the oxygen penetration range, it is possible to have aerobic/anoxic or aerobic/anoxic/anaerobic zones in the granule, resulting in a variety of microbial populations in aerobic granular sludge (AGS) (Liu and Tay 2004, Rollemberg et al. 2018). Granules allow to obtain a high concentration of biomass in a small space with the consequent possibility of treating wastewater with high organic load values. However, a limit of the process seems to derive from the minimum organic loading rate (OLR) required to obtain granule generation (2.5 kgCOD/m^3 per day) (Liu et al., 2003). Above of this value it results that the average size of the granules tend to grow with the increase of the applied

organic load. However, Long et al. (2015) observed that when the OLR increased to 18 kgCOD/m^3·d, the granules were disintegrated, and biomass washout occurred. At low OLR values, filamentous bacteria are more abundant, causing granule disintegration. While, AGS instability at high OLRs has been attributed to three main aspects: increased granule size and consequent disintegration due to carbon penetration inability, hydrolysis and protein degradation of the granule nucleus and loss of the microbial self-aggregation capacity due to protein concentration reduction in the extracellular polymeric substances (EPS) (Rollemberg et al. 2018).

Aeration intensity and upflow air velocity are considered as the most important parameters of shear force control. High aeration intensity is favorable for keeping the stability of aerobic granules not only by providing sufficient hydraulic shear force but also by inhibiting the overgrowth of filamentous bacteria and large granules (Rollemberg et al. 2018).

A short settling time acts as a selective pressure on the microbial community of the system, leading to the selection of bacteria that form large and compact aggregates that settle quickly; the remaining aggregates that settle more slowly are instead eliminated together with the effluent (Liu and Tay, 2004).

Furthermore, the excellent stability and capacity of granular systems to treat organic wastewater with high resistance to degradation and containing toxic substances has been demonstrated (Lin et al., 2003).

2.3. SEQUENCING BATCH BIOFILTER GRANULAR REACTOR (SBBGR)

To facilitate the reuse of treated wastewater in agriculture a simplified scheme treatment would be required. An innovative technology named SBBGR (Sequencing Batch Biofilter Granular Reactor), developed by the Water Research Institute (IRSA) of the Italian National Research Council (CNR) could comply with this request. SBBGR combines the advantages of periodic system (i.e., greater flexibility and stability) whit those of attached biomass system (i.e., greater robustness and compactness). In fact, like GSBR described in par 2.2.2.4, SBBGR belongs to the family of granular reactors operating in discontinuous mode. Moreover SBBGR contain a mixed biomass constituted of granule and biofilm growing attached and among the pores of a filling material.

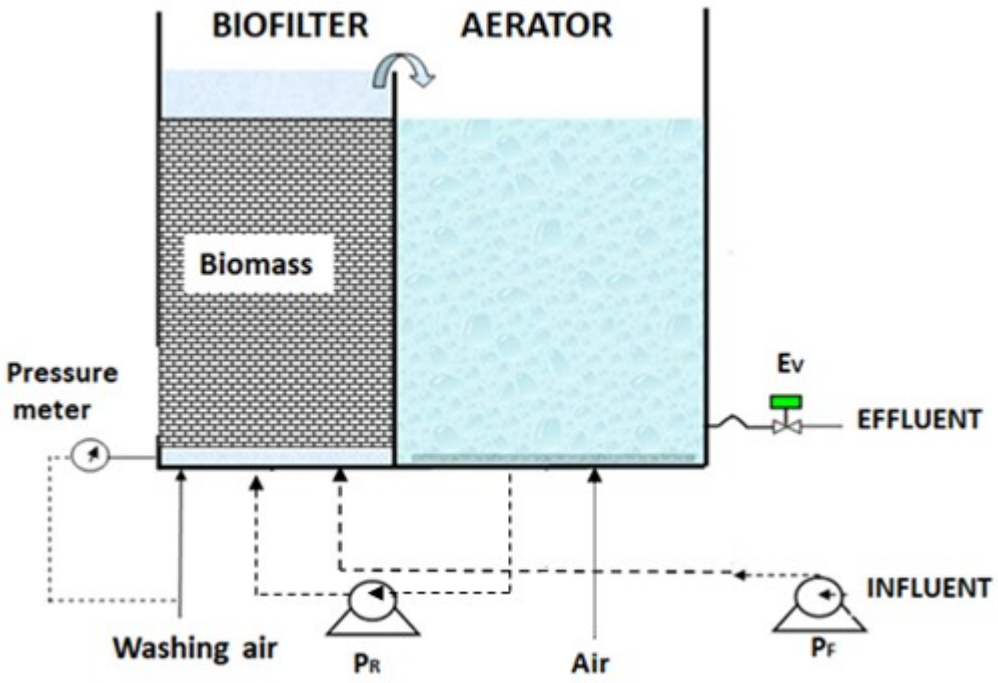

Fig. 2.3.1. Typical SBBGR system scheme

SBBGR technology could be applied directly to the raw wastewater, thus bypassing the primary treatments (primary sedimentation), replacing the common activated sludge sys-

tems (i.e., as secondary treatment) and, due to its operating characteristics and biomass type, avoiding the need for a secondary settler.

The SBBGR system consists essentially of a single unit in which the wastewater is introduced, treated (by selected microorganisms) and extracted sequentially. In SBBGR we can distinguish two compartments: the biofilter and the aerator (figure 2.3.1.). The biofilter is filled with plastic material, confined in a space delimited by two plates, called bed, in which biological oxidation processes are developed. The aerator provides to contain the waste to be treated and to oxygenate it with the air supply in the liquid phase, a recycling pump circulates the wastewater from the aerator to the biofilter to improve the distribution of the substrate and oxygen along the height of the bed allowing biological degradation processes; a motorized valve allows discharge of the effluent from the aerator at the end of the treatment cycle. The operation of an SBBGR system is extremely simple and is based on the succession of treatment cycles, the particular conformation of the SBBGR reactors means that the normal cycle of operation of these systems is composed of only three phases: feeding, recirculation and discharge of the effluent (figure 2.3.2.). Thanks to the granular biomass confined in the bed and separated from the effluent, the SBBGR system is not affected by the problems of biomass sedimentability and does not require a sedimentation phase, the lack of this phase represents a first advantage over common SBR systems. A pump (P_F in figure 2.3.1.) injects the waste into the biofilter in up-flow mode during the filling (or loading) phase. Once the reactor is fed with a pre-set volume of liquid, the recirculation pump (P_R in figure 2.3.1.) is activated, which makes the wastewater and the oxygen flow through the biofilter bed, where the polluted waste is removed by biomass (reaction phase). SBBGR is a unique system in virtue of the particular type of biomass growing in it: a mixture of biofilm and granules packed in a filling material (Di Iaconi et al.,2014). Air can be provided by using an aerator during the entire reaction phase or at time fraction, creating anoxic phase. When the concentrations of the various pollutants have been reduced to a satisfactory level, the recirculation pump and the aerator stop and the activation of a motorized valve (E_V in figure 2.3.1), allows the discharge by gravity of the treated effluent (drawing phase). When the effluent has been completely removed from the system, the plant is ready to start a new treatment cycle equal to the previous one. All this is made possible thanks to the use of an automatic control system, based on microprocessor and timer, which allows the various phases of the treatment cy-

cle to be alternated, and the managment of the operation of the various devices connected to the system (P_F pump, P_R pump, aerator, E_V valve).

A pressure meter, positioned on the bottom of the biofilter, continuously measures the head losses due to the growth of the biomass and the solids, present in the wastewater, retained by filtration. Upon reaching a fixed set head loss value, the washing operation (performed with compressed air only) is carried out, in this way the head losses fall within a range of values that ensures the correct functioning of the system.

The high porosity of the filling material used and the characteristics of the biomass that develops (high density and compactness) make it possible to significantly lengthen the duration of the service phase, considerably reducing the frequency of the washing operations compared to what is done with normal biofilters. These conditions also favor a substantial increase in the concentration of biomass inside the biofilter (up to an order of magnitude greater than those present in traditional treatment systems) with a consequent reduction in sludge production (due to high sludge age).

The SBBGR technology does not require particularly complicated air diffusers, which are usually subject to frequent malfunctions, since the service air is insufflated in an area outside the filling material and conveyed by the recirculation stream to the confined biomass in the biofilter. Moreover, it is possible to increase the treatment capacity of the system by dosing air and/or pure gaseous oxygen in the recirculating liquid stream, thus obtaining a dissolved oxygen concentration greater than that of air saturation. It is important to underline that pure oxygen is used in a specific way (i.e. only to increase the concentration of dissolved oxygen in the already aerated wastewater) and is controlled since the dosage point allows its complete dissolution and use.

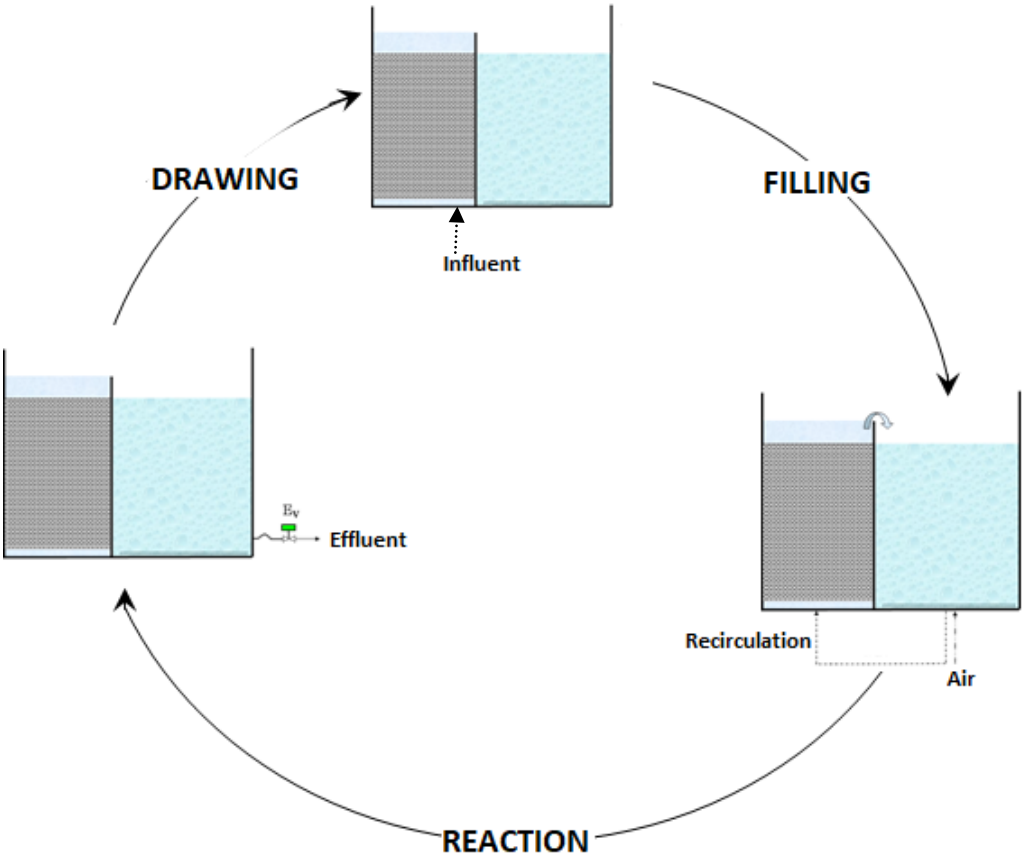

Fig. 2.3.2. SBBGR treatment cycle.

2.3.1 Granulation process in SBBGR system

The potentiality of the system is due to the particular type of biomass that develops (mixture of biofilm and granules with high density, confined in the plastic porous medium of the biofilter) thanks to the particular operating conditions used and which make the system unique in its kind.

The granule formation mechanism in SBBGR reactors takes place through 4 distinct phases (figure 2.3.3). During the first phase the activated sludge used as inoculim produces a thin biofilm completely covering the surface of the carrier material. Later, an increase in biofilm thickness is recorded which therefore led to an increase in biomass con-

centration. Subsequently, a detachment of biofilm particles and their deposition inside the pores of the filling material is observed. The particles entrapped inside the carrier continue to grow, reaching a size and shape similar to granules. Once formed, granules remain trapped inside the pores of the filling material.

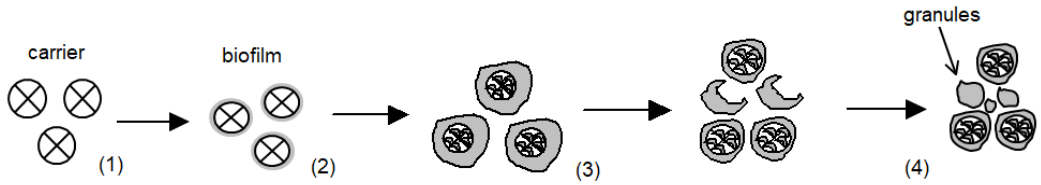

Fig. 2.3.3. Sketch of the granules generation steps during the start-up period (Di Iaconi et al. 2005).

Therefore, at the end of the start-up period (i.e., a gradual process of several months, variable according to the plant scale), the biomass present in the reactor bed consists of two different fractions: the biofilm attached to the carrier and the granules confined in the interstitial pores of the filling material (De Sanctis et al., 2010). The granules have a density that is 4 to 5 times greater than that of activated sludge systems (Figure 2.3.4.).

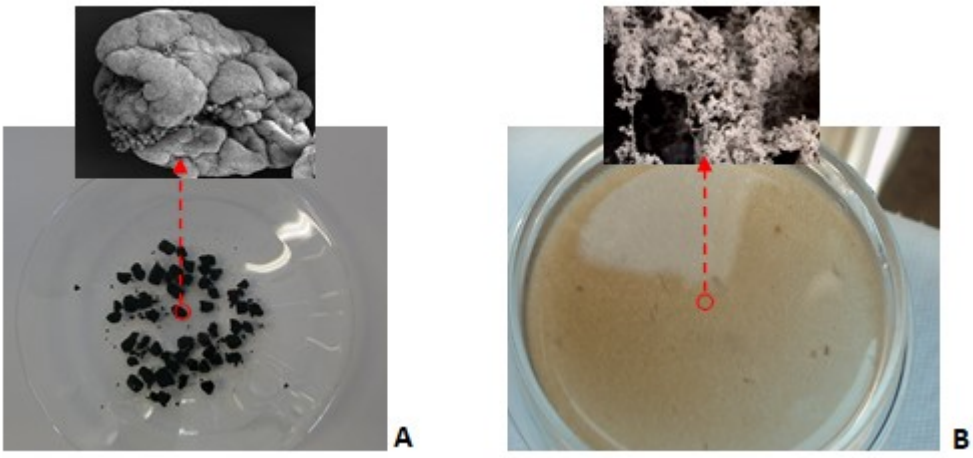

Fig. 2.3.4. Photo with relative magnification (100X) of a sample of granular biomass (A) and one of activated sludge (B).

To allow the formation and retention of the granules in the filling material it is important to use a packing material that guarantees both a high initial porosity of the bed and a optimal pore size. Such a packaged bed makes it possible to obtain a higher concentration of biomass, even up to 50 kg/m^3_{bed} (ie more than an order of magnitude greater than that found in conventional biological systems).

The increase in biomass concentration in the SBBGR system is a strategy applied to limit the production of excess sludge. In fact, as is known, the net yield of biomass growth, Y_n, is given by the difference between the growth linked to the consumption of the substrate and the decay (eq. 2):

$$Y_n = Y - bX(-dS/dt) \qquad \textbf{(Eq. 2)}$$

Y is the thermodynamic yield of growth (that is the one linked to the consumption of substrate); b is the decay rate, which takes into account all the factors responsible for the decrease in concentration of biomass (endogenous metabolism, death, lysis and predation); *X (-dS/dt)* is the inverse of the consumption rate of the substrate per unit of biomass.

Therefore, as the concentration of biomass increases within the reactor, the net growth yield decreases.

In traditional activated sludge systems, this strategy is partially hampered by the fact that the concentration of biomass can not be increased beyond a certain value due to its low separation speed from the liquid phase in the secondary settler.

The formation of stable and dense aerobic granul sludge is also based on the feast-famine regime, typical of periodic reactors. A periodic system cyclically carries out the operations of feeding, treatment and discharge of the wastewater. This operation, if well managed, leads to the alternation of "feast and famine" conditions. In fact, the reactor loading phase is characterized by high concentrations of readily biodegradable substances, which cause the biomass to store them in the form of storage polymers. In the famine phase (i.e. when the exogenous substrate has been completely used), the microorganisms use the polymers previously stored for growth and maintenance.

Granulation is favored by a decrease in the growth rate of micro-organisms, the famine phase is usually characterized by a low net yield of biomass growth since the micro-organisms must "depolymerize" the stored material before using it; this involves a waste

of energy. A period of rather prolonged famine, however, undermines the stability of the granules.

The role of shear forces is extremely important for the development of the biofilm, in fact a dense and compact film is formed when the detachment rates are greater than the production of new biomass (van Loosdrecht et al., 1995). In literature several studies report the relationship between shear forces and the density of biomass (Chang et al., 1991; Ohashi et al., 1994; Kwok et al., 1998), shear forces also contribute to the generation of granular biomass, determine the shape of the three-dimensional structures formed and regulate the surface. High values of shear stress compact the microbial aggregates in granules, as the microorganisms are pushed, to "escape" the adverse fluid-dynamic conditions, towards the inner layers of the biomass, thus colonizing all the empty spaces available; obviously this leads to an increase in density.

Furthermore, the increase in shear stresses stimulates the production of EPS making the surface of the microorganisms that populate the outer part of the granule more hydrophobic in order to reduce friction with the liquid phase (Liu et al., 2002). The EPS contribute mainly to the aerobic granules stability, and literature shows these polymers function as a “biological glue” for granule formation and stability (Rollemberg et al. 2018). In the SBBGR system the aeration takes place in a compartment separated from the biofilter, so the main parameter of the shear force control is the flow rate of wastewater in the biofilter, which depends on the flow rate of the recirculation pump and the porosity of the bed.

Another parameter that can influence the structure of the granules is the concentration of dissolved oxygen. In GSBR systems it has been estimated that the lower is the concentration of dissolved oxygen the greater is the diameter of the granules, but obviously we must consider that the oxygen must be at a concentration that guarantees nitrification (Mosquera-Corral et al. 2005).

In SBBGR process characteristics and granule conformation allow simultaneous nitrification-denitrification in mature granules and led to a high nitrogen removal (De Sanctis et al., 2009). The presence of these microorganisms in the reactor contributes to the structure and stability of the granule. In fact, denitrifying bacteria are characterized by a lower growth rate than other bacteria because in famine conditions they obtain energy from stored polymers; furthermore nitrifying bacteria are autotrophic organisms and therefore classified as slow-growing microorganisms. Their high presence in SBBGR biomass is at-

tributable to the high value of the sludge age of the system (over 120 days). In fact, nitrifying bacteria in traditional treatment plants (characterized by sludge age values generally between 1 and 3 weeks) are present only in very low concentrations.

The considerable increase in sludge age is due to the high density and concentration of granules, the microorganisms spend much time in the endogenous metabolism phase where the biomass decay rate is high, and thus the biomass production rate is low, as a result, a reduction in sludge productionis obtained (Di Iaconi et al., 2010).

2.4. SCOPE OF RESEARCH

One of the most promising systems based on granular biomass are Sequencing Batch Biofilter Granular Reactor Technology (SBBGR). In this system, the granules are not suspended as in Sequencing Batch Granular Reactor (SBGR), but retained in the pores produced by packing the reactor with a filling material.

The advantages of SBBGR technology can be summarized as follows:

- has high purification capacity, with a consequent reduction in reaction volumes;
- does not require a secondary settler;
- produces a small quantity of sludge;
- is very compact, as all the phases take place in a single operating unit,
- shows great operational flexibility, a fundamental requirement for the treatment of urban wastewater, which is characterized by a high variability in terms of flow rate and composition.

SBBGR overcomes some of the frequent drawbacks associated with traditional biological processes and improve the treatment line. On the basis of the above, the interest in applying the SBBGR technology directly on the raw wastewater fof treating and reusing purified sewage appears evident.

The aim of this research activity is to test a simplified treatment scheme compared to the traditional ones, in which the main unit is the SBBGR system.

This treatment scheme, compact and flexible, turns out to be a possible treatment solution for agriculture reuse. Water could be reused near the same area where is consumed, meeting water agriculture demand and also increasing freshwater resources for other needs.

Therefore, this thesis work reports the results obtained during a pilot scale experimentation aimed at evaluating the effectiveness of SBBGR technology for the treatment and reuse of a raw urban wastewater. The effectiveness of the treatment was assessed in terms of the removal of the main parameters characterizing urban wastewater (ie, COD, suspended solids, TKN, ammonia, nitric nitrogen, nitrous nitrogen and phosphorus).

As is known, the reuse of wastewater in agricultural irrigation requires specific levels of effluent quality in order to avoid serious risks to the environment and public health. In the present study, the capability to produce an effluent suitable for agriculture according to

stringent parameters of the Italian legislation has been evaluated for SBBGR system and also SBBGR enhanced with sand filtration and chemical (by peracetic acid, PAA) or physical (by UV radiation) disinfection.

In this thesis all physical and chemical parameters indicated by the Italian regulation are monitorated. Differently, the analysis of microbiological parameters was not limited to the two parameters required by this regulation (*E. coli* and *Salmonella*). Indeed, the microbial indicators were chosen considering that most human pathogens that could be derived from the reuse of wastewater belong to the domains of bacteria, viruses and protozoa, and these microorganisms are characterized by different physiological characteristics and consequently different survival rates in wastewater treatment. Therefore, microorganisms belonging to these domains were selected to evaluate the quality of the treated-wastewater. The selected microbial indicators were total coliforms, *E. coli and Salmonella* (representative of bacteria), *Clostridium perfringens* spores (representative of spore-forming bacteria*), Somatic coliphages* (representative of viruses) and *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts (representative of protozoa).

Moreover, one of the emerging problems concerning water pollution due to anthropic action is the presence and diffusion of antibiotic resistance genes (ARGs) and WWTPs have been specifically individuated not only as the main sources of antibiotics, but also as platforms for the spread of ARGs through the environment (Rizzo et al., 2013). Thus, the assessment of ARGs reduction efficiencies of the proposed wastewater treatment has been included among the objective of this thesis.

2.5. MATERIAL AND METHODS

2.5.1. Experimental pilot-plan and operational program

In the present thesis study a pilot-scale SBBGR ptototype was used, with a total volume of about 300 liters and a height of about 3.2 meters (Figure 2.5.1.). The biofilter is the reactive part of the plant as it contains the biomass, and reactions take place there. In the prototype used, it consisted of a cylindrical steel reactor, having a volume of about 120 liters (diameter: 22 cm, height: 3.2 m), filled with plastic material in bulk (plastic elements in the shape of wheel known under the trade name kmt-k1 of Kaldness, features: 7 mm height, 11 mm diameter, 650 m^2/m^3 specific area, 0.95 g/cm^3 density, 0.7 bed porosity and 50-80 mm^3 voids size) (Figure 2.5.2.).

Fig. 2.5.1. A photograph of the SBBGR treatment plant.

This material was packed between two perforated plates that delimited a zone commonly called bed. The aerator consisted of a further steel cylindrical compartment, having a volume of about 180 liters (diameter: 27 cm, height: 3.5 m). Its function was to provide the air necessary for the process (aerobic process) by means of a blower connected to a perforated plate, capable of forming fine bubbles, positioned on the bottom of the aerator.
Separating the reactor in which the biomass is present from the one where the air supply takes place has the advantage of not having to install complex ventilation systems, instead required by traditional technologies (where the air is supplied in the same unit that contains the biomass). This avoids a common problem encountered in conventional systems: biomass that tends to clog the holes of the devices used for air supply.
The biofilter and the aerator were hydraulically connected at the bottom by a volumetric pump (recirculation pump), which ensured the continuous recirculation of the oxygenated effluent from the aerator to the biofilter (through the biofilter packing material), and at the top due to a pipe that allowed the wastewater that had passed through the biomass, to return by fall into the aerator to be oxygenated again.

Fig. 2.5.2. Filling material kmt-k1 of Kaldnes.

The operation of the pilot plant was based on six-hours treatment cycles, each organized in three consecutive phases: loading (30 minutes), reaction (5 hours) and discharge (30 minutes). During the loading phase, a pre-set volume of wastewater to be treated (60 L of

influent) was introduced by a peristaltic pump (loading pump, max flow: 120 L/h) into the biofilter. Subsequently (reaction phase) there was the activation of the blower (flow rate: 5 Nm^3/h) and of the recirculation pump (flow rate: 140 L/h) which made the oxygenated waste flow from the aerator to the biofilter. During the recirculation phase, air is provided by means of discontinuous blowing periods which start every 5 min and last for 25 min. Finally, during the unloading phase there was the removal of the treated wastewater (effluent of the plant) by gravity through the opening of a motorized valve, which put the aerator in communication with the outside. Once the effluent was discharged, the plant was ready to start a new treatment cycle equal to the previous one.

The plant was completely automated thanks to the use of a PLC (Programmable Logic Controller) that allowed the alternation of the various phases of the treatment cycle. In addition, the plant was equipped with a touch screen monitor that allowed to be able to "converse" with the PLC, or to manage the duration of the various phases of the treatment cycle, changing the timing of intervention of the equipment (feed pump, pump recirculation, blower and exhaust valve).

A meter connected to a pressure probe, positioned on the bottom of the biofilter unit, continuously measured the values of head losses. Upon reaching a determined head loss value, a washing step was carried out with compressed air in order to remove excess biomass from the biofilter bed. The washing operation continued until the value of the pressure was within a range that guaranteed the correct operation of the system. The biomass detached from the biofilter bed was completely recovered, through the opening of a valve positioned on the bottom of the biofilter, and characterized in terms of volume, total suspended solids and volatile suspended solids in order to be able to determine the production of sludge and its grade of stabilization.

The pilot plant was fed with a raw urban wastewater coming from small villages in the district of Bari which was supplied daily to the plant by means of a truck.

The data reported in this thesis do not include the start-up of the plant during that period the organic load of the SBBGR was gradually increased in order to allow the generation of granular biomass in the plant, according to the experience gained during previous studies (Di Iaconi et al. 2005, De Sanctis et al. 2010).

The experimentation period reported here refers to 8 months at the end of 4 years in which the SBBGR plan had already been in operation for treating urban wastewater,

therefore the reported data refer to a system in stable and stationary conditions. During this period the organic load applied to the plant was found to be about 1kg COD/$m^3_{biofilter}$·d, as a function of the concentration value of the COD in the incoming wastewater and the hydraulic load on the system.
In order to evaluate the performance of the plant, in terms of efficiency of removal of the main parameters, samples of influent and effluent were taken and analyzed, with a frequency of 1 time a week.

2.5.2. Sand filter and disinfection strategies

The sand filter consisted of a plastic cylindrical unitwith a volume of about 120 L, partially filled with river sand supported by gravel as follows: 10 cm of gravel (irregular shape; larger diameter: 5–15 mm), 40 cm of sand (diameter: 0.3–0.8 mm), 5 cm of gravel on the filter top to promote uniform wastewater distribution (Table 2.5.1). The flow rate through the filter was checked a minimum of two times a week. Due to the filter clogging, a washing step was carried out when the flow rate decreased under 10 L/h.

Sand filter	
Volume (L)	120
Diameter sand filter (cm)	35
Sand bed high (cm)	55
Lower gravel layer height (cm)	10
Sand layer height (cm)	40
Upper layer gravel height (cm)	5
Flow rate (L/h)	≥10

Tab 2.5.1. Main features and operating conditions of sand filter

Tertiary disinfection tests were carried out on the SBBGR effluent by using two strategies: physical disinfection by UV radiation or chemical disinfection by the addition of peracetic acid (PAA).
The UV disinfection was conducted with a flow-through annular photoreactor (volume: 0.5 L) equipped with a low pressure mercury vapour UV lamp (MTL844-G model by Helios

Italquartz srl Milano, Italy) with an emission peak at 254 nm (input power: 40W). UV fluence rate, determined by uridine actinometry (Jin et al., 2006), was 40 mW/cm^2. The plant effluent was collected and flowed through the UV lamp by means of a submerged pump having a flow rate of 1800 L/h in order to obtain an exposure time of 1 s. Therefore, a UV dose (obtained by multiplying the fluence rate by exposure time) of 40 mJ/cm2 was applied.

Chemical disinfection tests were carried out by adding a known volume of a commercial PAA solution (15 g PAA/L and 10 g H_2O_2/L, OXIFIBRO, Nuova Farmec, Italy) to a fixed volume of SBBGR effluent. The solution was maintained under stirring for 30 minutes and then the microbial analysis was performed. Considering the high cost of PAA and the increase in COD concentration caused by its addition to the effluent, 1 mg/L PAA concentration was tested. Taking into account the low PAA concentrations used, the residual disinfectant was not quenched by the addition of sodiumthiosulphate (for PAA removal) and bovine catalase (for hydrogen peroxide removal) as reported in the literature. (Rossi et al., 2007).

2.5.3. Analytical methods and procedures

Organic matter

The characterization of organic substrates was carried out with the COD test. The test was performed on raw and filtered (filter porosity = 0.45 μm) waste samples, to obtain the total and soluble COD concentration, respectively.

As known, the test determines the amount of oxygen consumed by the chemical oxidation of organic and inorganic substances contained in the sample.

The method is based on the reaction in an acid environment - by addition of sulfuric acid - and at a temperature of about 145 °C, using potassium dichromate as oxidizing agent and silver sulfate as a catalyst, according to the unbalanced reaction:

$$\underset{\text{organic matter}}{C_aH_bO_c} + Cr_2O_7^{2-} + H^+ \rightarrow Cr_3^+ + CO_2 + H_2O$$

The concentration of oxidizable organic and inorganic substances, for the conditions of the method, is proportional to the amount of potassium dichromate consumed. The chloride ion is considered an interferent, since its oxidation can occur in such conditions, therefore, chlorides are masked with mercury sulfate. Operationally, the COD was determined by cuvette test (LCK 314, Hach Lange), digestion for 2 hours at 148 °C in the HT 2005 DR LANGE digester and reading with the DR 2800 Hach Lange portable spectrophotometer, after sample cooling to room temperature. The spectrophotometer performs analyzes on "cuvette - test", marked with a barcode, which is identified automatically thanks to the integrated barcode reading system (IBR) with which the instrument is equipped. The final reading of the sample is performed colorimetrically, as the dichromate is yellow, while the Cr^{3+} is green. In this specific case, the yellow color of Cr^{6+} is read photometrically.
Determination of 5 day biochemical oxygen demand (BOD_5) was determined by cuvette test (LCK 555) with Lange BioKit LZC555 used as inoculation material. In order to completely remove chlorine, its by-products or other interfering matter, the supply water used for the creation of the dilution water must be aerated for at least 2 days and enriched withtrace element. Once the inoculum solution has been prepared with the sample, the cuvettes are incubated in a temperature chamber at 20 ° C for 5 days. Nitrification is inhibited by 5 mg/L allylthiourea. The dissolved oxygen is analysed in an alkaline solution with a pyrocatechol derivative in the presence of $Fe^{2+,}$ under which conditions a red dye is formed.

Nitrogen compound
720 °C catalytic thermal decomposition/chemiluminescence methods are adopted for total soluble nitrogen measurement on filtered samples (filter porosity = 0.45 μm) with TN (Total Nitrogen) Unit of TOC analyser (TOC-L Shimadzu). Total nitrogen (TN) on raw samples was also determined by LCK cuvette test 238 Hach Lange. This test is based on the following principle: organic and inorganic nitrogen is oxidized to nitrate by digestion with peroxidisulphate. The nitrate ions react with 2,6 - dimethylphenol in an acid medium to form nitrophenol, the concentration of which is determined photometrically.
TKN (Kjeldahl nitrogen) was calculated from the difference between total nitrogen (TN) and oxidized nitrogen (sum of nitric and nitrous nitrogen).

The determination of nitrous and nitric nitrogen was performed on samples of filtered wastewater (filter porosity = 0.2 µm) using an ion chromatograph (Thermo Scientific™ Dionex™ Aquion™ Ion Chromatography System).
Ammonia was determined with UV400 Online Water Analyser (Tethys instrument). The method is based on the measurement of the absorption spectrum of gaseous ammonia (NH_3) in equilibrium with the ammonia in liquid phase (NH_4^+) present in the sample by Fourier transform. The equilibrium of the chemical reaction between ammonia and ammonium ions is displaced by the addition of small amounts of a concentrated solution of sodium hydroxide (NaOH): when pH value higher than 11 is reached, the concentration of ammonium ions is negligible compared to that of ammonia in the gas phase (less than 0.1%).

Solids
For the determination of total suspended solids (TSS), a filtration with a membrane filter (diameter = 90 mm, porosity = 0.45 µm) was performed and subsequent drying in an oven at a temperature of 103-105 °C. The concentration of total suspended solids is calculated as follows (eq. 3.):

$$TSS\ (mg/l) = \frac{(M_1 - M_0) \cdot 1000^2}{V} \qquad \textbf{(Eq.3.)}$$

M_1 is the weight, expressed in grams, of the filter and of the residue after drying;
M_0 is the weight, in grams, of the filter;
V is the volume, in ml, of the sample subjected to filtration.

Volatile suspended solids constitute the organic fraction of the TSS. They have been determined as a loss to incineration, as a difference between the TSS and the fixed residue, represented by the solids that remain on the filter after incineration, conducted in a muffle-furnace at a temperature of 560 °C for 2 hours.
The concentration of volatile suspended solids is calculated as follows (eq 4.):

$$VSS\ (mg/l) = \frac{(M_1 - M_2) \cdot 1000^2}{V} \qquad \textbf{(Eq. 4.)}$$

M_1 is the weight, expressed in grams, of the filter with sample residues after drying;
M_2 is the weight, in grams, of the filter with the residues after incineration;
V is the volume, in ml, of the sample subjected to filtration.

Phosphorus

In water phosphorus is generally present as ortho-phosphate, polyphosphate and organic compounds. In wastewater analysis, phosphorus concentration is always expressed in terms of phosphorus content in the form of phosphate. The result is indicated as $P\text{-}PO_4$. The determination of phosphorus was carried out using a cuvette method LCK 349 Hach Lange, which exploits the following principle: the polyphosphate and organic phosphorus forms are hydrolyzed to orthophosphates by boiling in a strong acid solution. In acidic solution with molybdate and antimony ions, phosphate ions form an antimonylphosphomolybidate complex which, with ascorbic acid, is reduced to phosphomolyibidate blue, the concentration of which is determined photometrically.

Conductivity and pH

Conductivity is measured with a probe as the electrical conductivity of all ions dissolved in water, both positive and negative (Ca^{++}, Na^{+}, NO_3^{-}, etc.).
The pH of the samples was measured potentiometrically using a glass electrode combined with a suitable reference electrode. The value to be determined was obtained after carrying out a calibration operation with two buffer solutions at known pH, brought to the same temperature of the sample.

SAR

To better evaluate the suitability of the treated wastewater for agricultural use, the sodiumadsorption ratio (SAR) in the plant influent and effluent was monitored six times during the experimental campaign. This parameter expresses the relative activity of sodium ions in the exchange reaction with the soil according to the following equation 5:

$$SAR = \frac{Na^{+}}{\sqrt{1/2\,(Ca^{2+} + Mg^{2+})}} \qquad \textbf{(Eq. 5.)}$$

High concentrations of ions in water affect the permeability of soil and cause infiltration problems. This is due to the fact that sodium is able to replace calcium and magnesium in the soil, leading to the dispersion of soil particles. The soil becomes hard and compact when dry and reduces infiltration rates of water and air into the soil, affecting its structure.
The analysis of sodium, calcium and magnesium was carried out by means of atomic absorption spectrophotometer analysis.

2.5.4. Microbiological parameters

Total coliforms and *Escherichia coli*

Total coliforms are a group of rod-shaped, gram-negative, aerobic and facultative anaerobic non-spore-forming microorganisms that ferment lactose with gas and acid production. Since they are present in faecal material of human origin with an average density of 10^9 CFU/g, have been considered for decades indicators of water contamination together with fecal streptococci. However, it is now known that the group includes environmental species that are able to colonize water, soil and vegetation.
The most recent studies distinguish the microorganisms included under the name of "Total Coliforms" in two main categories: the first, well known, is that of coliforms of faecal origin, which includes some species of the genera *Escherichia*, *Enterobacter*, *Citrobacter* and *Klebsiella*, present in faecal material of humans and warm-blooded animals and in contaminated waters and soils; the second corresponds to species that, on the contrary, are widely distributed in the environment, where they can also multiply, colonizing soil, water and vegetation.
The belonging to the group of coliforms, more than on the systematic characteristics of the various microorganisms, has historically been based on the method used for their detection, which exploits the ability to ferment the lactose with the production of gas and acid at the temperature of 35 ÷ 37 ° C in 48 hours. In recent years, it has been discovered that a high percentage of these microorganisms (around 99%) possess the β-D-

galactosidase enzyme, based on this discovery, new methods have been developed for the identification of Coliforms.

Escherichia coli is a rod-shaped, gram-negative, aerobic and facultative anaerobic non-spore-forming microorganism, which grows up to a temperature of 44.5 °C, lactose-fermenting, of fecal origin. The World Health Organization, for over a decade, has recognized the *E. coli* species as the primary indicator of faecal contamination of water; the US EPA studies have also contributed to support the need to replace, for the evaluation of water quality, the parameter "fecal coliforms" with the "*Escherichia coli*" parameter. This choice, now accredited by the entire international scientific community, is motivated by the clear predominance of *E. coli* compared to the other Coliforms in faecal material and by the lower sensitivity of the microorganism to the disinfection procedures compared to the majority of enteric pathogenic bacteria.It is the reference indicator in the legislation of many European countries. Several studies have consolidated the evidence that a high percentage of *E. coli*, around 98%, and with the exception of the serotypes O157: H7, possesses the β-D-glucuronidase enzyme. Therefore, substrates were formulated for the direct research of *Escherichia coli*, all based, no longer on the traditional reaction of lactose fermentation, but on the detection of the enzymatic activity of ß-D-glucuronidase, evidenced by the hydrolysis of ß-glucuronides chromogenic or fluorogens, with the release of colored or fluorescent compounds.

Colilert-18 assay by IDEEX was used for coliform analyzes. This method allows the simultaneous detection of total coliforms and *Escherichia coli*. Its use is rather simple and fast; it can be used to monitor all types of water samples, including those with suspended material.

The procedure provides (Figure 2.5.4.):

- the mixing of the reagents, packaged in monodose, with the samples and their addition in the incubation plates;
- the sealing of the plates and their incubation for 18 h at a constant temperature of 35 °C
- the count of positive wells (UV lamp is necessary to detect *E. coli* positive wells)
- the calculation of the concentration of the microorganisms present by consulting the specific table.

Fig. 2.5.4. Colilert-18 procedure.

The method is based on the probability principle used in the MPN Procedure, but thanks to the simultaneous analysis of a large number of wells (49 large wells and 48 small wells) is able to provide greater accuracy and repeatability.

Colilert-18 has become the new standard 9308-2: 2012 of the International Organization for Standardization (ISO). It is approved by the US Environmental Protection Agency (EPA) and is included in the Standard Methods for Examination of Water and Wastewater. Provides counts from 1 to 2,400 MPN/100 (mL Quanti-Tray/2000). Colilert-18 uses the ONPG and MUG nutrient-indicators with defined substrate technology (Defined Substrate Technology®, DST®) to detect coliforms and *E. coli*. Coliforms use the β-galactosidase enzyme to metabolize ONPG, which shift well colour from colorless to yellow (figure 2.5.5.), while *E.coli* uses the β-glucuronidase enzyme to metabolize MUG, creating fluorescence (figure 2.5.6.).

The method was applied on samples of plant influent (raw sewage), biological effluent, effluent after sand filtration, effluent after UV disinfection, effluent after disinfection with peracetic acid.

Fig. 2.5.5. Coliforms uses their β-galactosidase enzyme to metabolize ONPG and change it from colorless to yellow (thermalindo.com).

Fig. 2.5.6. *E.coli* use β-glucuronide enzyme to metabolize MUG and create fluorescence (thermalindo.com).

Clostridium perfringens

Clostridium perfringens is a gram-positive, rod-shaped, anaerobic, sulphite-reducing and sporogenous bacterium. Among the clostridia reducing sulphites is the species most frequently associated with feces of warm-blooded animals. It is present in human faecal material in concentrations ranging from 102 to 107 CFU/g, and is represented in dog and swine faeces, while it is less common, or even absent, in the faeces of other warm-blooded animals. Being a sporogenous microorganism it has longer survival times than the indicators of fecalization (*Escherichia coli* and enterococci). It is more resistant than coliforms and enterococci to disinfection treatments and can be used together with them as an indicator for pathogenic protozoa in water treatment to evaluate its efficiency.

To detect the presence of *Clostridium perfringens* spores the proposed method was adapted by Bufton,1959 and by the Italian methods for the detection of *C. perfringens* in water and wastewater (IRSA-APAT 7060-2003) based on the use of SPS agar (sulfite Polimixina Sulfadiazine Agar). It is a moderately selective medium for the isolation and counts of *C. Perfringens.*

SPS Agar contains:

- peptone 15 g (source of carbon, nitrogen, vitamins and minerals)

- 10 g yeast extract (provides B-complex vitamins that stimulate bacterial growth)
- B complex vitamins (stimulate bacterial growth)
- Ferric citrate 0.5 g, sodium sulphite 0.5 g (H_2S indicators)
- Polysorbate 80 (dispersing agent)
- Polymyxin B sulfate 0.01 g and sulfadiazine 0.12 g (inhibitors of organisms other than *Clostridium* spp.)
- Sodium thioglycolate 0.1 g (reducing agent)
- Agar 15 g (solidification agent)

Clostridia reduce sulphite, which reacts with ferric citrate iron to form a black precipitate of iron sulfide. The samples are subjected to a thermal shock of 80 °C for 10 minutes in order to remove all the microbiota and the vegetative forms of *C. perfringens*. Then, the sample is added to SPS agar and incubated in anaerobiosis at 44 ± 1 °C for 24 ± 1 hours. The presence of *C. perfringens* spores is indicated by black colonies resulting from the reduction of sulphite to sulfide, which reacts with iron salts (III) (figure 2.5.7.).

Fig.2.5.7. *C. perfringens* spores indicated by black colonies.

The calculation of the number of spores (present per mL of the original sample) is carried out by applying the following formula (eq. 6.):

$$x = \frac{N}{(n_1 V_1 F_1)+(n_2 V_2 F_2)} \qquad \textbf{(Eq. 6.)}$$

- x = concentration of spores *C. perfringens* per mL of original sample
- N = total number of typical colonies *C. perfringens* counted in tubes
- n_1, n_2 = number of replicas for the dilution F_1, F_2
- V_1, V_2 = test volume, in milliliters, used with dilution F_1, F_2
- F_1, F_2 = dilution factor (eg F = 1 for an undiluted sample, F = 0.1 for ten times dilution, etc.)

The method was applied on samples of plant influent (raw sewage), biological effluent, effluent after sand filtration, effluent after UV disinfection, effluent after disinfection with PAA. Each dilution tested was analysed in triplicate.

Salmonella

The *Salmonella* genus includes rod-shaped microorganisms belonging to the *Enterobacteriaceae* family. They are Gram-negative micro-organisms, facultative anaerobes, generally motile due to the presence of peritrichous flagella. They are classified according to serological characters that differentiate about 2000 types and serotypes. They are mainly characterized by the presence of two types of antigens: somatic (O) antigens, thermostable and resistant to the action of acids and alcohols, and ciliary (H), thermolabile antigens. *Salmonella* parasitize the intestine of man, domestic and wild animals; sometimes they can be isolated from the blood and internal organs of vertebrates. Gastric acidity is an important defense mechanism, but some species can survive and penetrate the intestinal epithelium, even if only *S. typhi* is systematically invasive. They can be responsible for widespread and ubiquitous diseases in humans, caused by ubiquitous serotypes widely used in farmed animals (minor salmonellosis). The disease usually manifests with diarrhea and enterocolitis of modest severity (except in the elderly, in the defedates, in the immunocompromised and in the children) and, generally, tends to a spontaneous healing.

However, many may be asymptomatic carriers. Systemic salmonellosis (typhus and paratifs), transmitted directly from human to human through the oro-fecal route, are instead caused exclusively by serotypes adapted to humans (*Salmonella typhi*, and *S. paratyphi A, S. schottmuelleri* and *S. hirschfeldii*). In general they are of modest gravity but, in the absence of adequate therapy, they can also be fatal.

The presence of *Salmonella* in the water environment unequivocally represents the existence of primary faecal contamination (direct intake of sewage discharges) or secondary contamination (washout of contaminated soils). The *Salmonella* are, in general, in a reduced number compared to the indicators of faecal contamination and variable according to the widespread diseases within the population. Their detection in the waters requires the examination of relatively high volumes of water. In Italy, *S. infantis*, *S. typhimurium*, *S. derby*, *S. veneziana* are the most frequently diffused serotypes and isolated from environmental sources. Salmonella was analysed according to the 7080 IRSA method. The method is based on four main steps that allow a determination of the presence/absence of *Salmonella* spp. The research is very sensitive but at the same time precise, in fact *Salmonella* spp. can be present in very low concentrations and associated with other microorganisms of the *Enterobacteriaceae* family, but it is nevertheless detected.

1. Pre-enrichment in non-selective media

Pre-enrichment is a necessary step to "awaken" the microorganisms that may have undergone stresses due to previous treatments of the water matrix. Peptonate water is added to the test sample at room temperature and then incubated for 18±2 hours a 36±2 °C.

2. Enrichment in selective medium

The culture obtained in the pre-enrichment phase is separated into two aliquots, one of which is inoculated in the Rappaport-Vassiliadis medium with soya (RVS broth), while the other one is inoculated into the Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn broth). RVS broth is incubated at 41.5±1 °C for 24±3 hours, while MKTTn broth is incubated at 37±1 °C for 24±3 hours.

3. Sowing and identification

Sowing is carried out on two solid soils: Xylose Lysine Deoxycholate agar (XLD agar) and a complementary medium that allows the identification of lactose-positive *Salmonella* (the most used is the solid ground Brilliant green agar, BGA). The XLD agar is incubated at

36±2 °C and examined after 24±3 hours, the other medium is incubated according to the manufacturer's instructions.

4. Confirmation of the colonies

The presumptive *Salmonella* colonies appear in both mediums as small circular colonies, with a central black zone and a transparent-reddish halo due to the color change of the indicator. The negative H_2S variants grown on XLD agar are pink and in the center they show a darker pink color. The positive lactose variants are yellow and may or may not have a darkening zone. The selected colonies are sown on agar solid medium enriched with nutrients to allow isolated colonies to develop. Then it is possible to proceed with the execution of confirmatory biochemical and serological tests (Triple Sugar Iron Agar, Urea Agar, Moeller Lysine Decarbo xylase Broth, Onpg Test, MR-VP Medium and Tryptone Tryptophan Medium) (7080: Metodi APAT IRSA).

Cryptosporidium and *Giardia*

The spherical protozoan *Cryptosporidium parvum* and the flagellated protozoan *Giardia lamblia* are etiologic agents of acute forms of gastroenteritis in humans. Both infections are transmitted by fecal-oral route through the ingestion of *Cryptosporidium* oocysts and cysts of *Giardia*. Infections with *Cryptosporidium* and *Giardia* are widely reported with one prevalence in industrialized countries of 1-3% for the former and 2-5% for the second and, in the developing countries, 5-10% for Cryptosporidium and 20-30% for Giardia (Masciopinto et al. 2011, 7130: Metodi APAT IRSA 2003). The most recent studies have shown that oocysts and cysts can be found in surface and deep waters, in marine environment and wastewater. The water was therefore recognized among the main vehicles of infection. Poor specificity of the host favors the spread of parasites in the environment, as well as the high number of cysts and oocysts emitted by infected animals and their extraordinary resistance to disinfection and environmental conditions (Masciopinto et al. 2011, Moreno et al. 2018)

The environment may become contaminated through direct deposit of human and animal feces, through run-off water of soils used for grazing infected animals or through sewage and wastewater discharges. Recognition of water as an important source of transmission

for humans has meant that *Cryptosporidium*, together with *Giardia*, are considered among the most widespread water-borne pathogens.

Pathogenic protozoan parasites, *Cryptosporidium* and *Giardia*, were detected by US EPA Method 1623. The analysis was conducted on 250 mL of influent wastewater and 5 L of treated wastewater respectively. The results were expressed in terms of oocysts/L and cysts/L respectively. Test procedure provides different steps as summarized:

- Water sample is filtered and the oocysts, cysts, and extraneous materials are retained on the filter.
- Materials on the filter are eluted and the eluate is centrifuged to pellet the oocysts and cysts, and the supernatant fluid is aspirated.
- The oocysts and cysts are magnetized by attachment of magnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies. The magnetized oocysts and cysts are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. The magnetic bead complex is then detached from the oocysts and cysts.
- The oocysts and cysts are stained on well slides with fluorescently labeled monoclonal antibodies and 4',6-diamidino-2-phenylindole (DAPI). The stained sample is examined using fluorescence and phase contrast microscopy.
- Qualitative analysis is performed by scanning each slide well for objects that meet the size, shape, and fluorescence characteristics of Cryptosporidium oocysts or Giardia cysts. Quantitative analysis is performed by counting the total number of objects on the slide confirmed as oocysts or cysts.

Enteroviruses and adenoviruses

Viruses excreted with feces or urine from any species of animal may pollute water. Especially numerous, and of particular importance to health, are the viruses that infect the gastrointestinal tract of man and are excreted with the feces of infected individuals. These viruses are transmitted most frequently from person to person by the fecal-oral route. However they also are present in domestic sewage which, after various degrees of treatment, is discharged to either surface waters or land. Consequently, enteric viruses may be present in sewage-contaminated surface and ground waters that are used as sources of drinking water. The viruses known to be excreted in relatively large numbers with feces include Reoviruses, Rotaviruses,Norovirus, the hepatitis A (infectious hepatitis) virus, Po-

lioviruses, Coxsackieviruses, Echoviruses, and other Enteroviruses and Adenoviruses (APHA, AWWA,WEF, 2005).

Analyses of enteroviruses and adenoviruses were conducted in 1 L of influent wastewater and 10 L of treated wastewater. Sample concentration was performed by means of Virosorb Zeta Plus 1MDS filters (CUNO-3M); filters were eluted in beef extract (3%) and purified with PEG 6000 as described in Masciopinto et al. (2011). Enteroviruses were quantified by RT-QPCR as reported in Masciopinto et al. (2011) Viral RNA was extracted using TRIzol LS Reagent (invitrogen by life technologies), Enterovirus cDNA was synthesized by reverse transcription, using specific primers, and successively amplified by means of two PCR reactions that produced 155 base pair (bp) amplified products for Enteroviruses. The specificity of the RT-PCR reaction in the positive samples was confirmed by sequencing the obtained amplified DNA sequences. Sequence data were analyzed and compared to collections of data available online (http://www.ncbi.nlm.nih.gov/). The software package ClustalW2 (www.ebi.ac.uk) was used for the alignment. The presence of Enterovirus in the positive samples was determined using real-time RT-PCR with SYBR Green stain. An Enterovirus calibration curve was obtained using serial dilution (from 105 to 101 genomic copies (gc)/µL) of a commercially available Enterovirus RNA standard (Armored RNA ENTEROVIRUS) (Masciopinto et al. 2011). Adenoviruses were analysed as described in Wyn-Jones et al. (2011) using primers Hex1deg and Hex2deg for the first round of amplification and primers nehex3deg and nehex4deg for the second round. The amplicons were electrophoresed in a 2% agarose gel stained with 10 $ng \cdot mL^{-1}$ ethidium bromide or equivalent nucleic acid staining methods such as SYBR-Gold, and subsequently visualised by UV transillumination (Wyn-Jones et al. 2011).

Somatic coliphages

Somatic coliphages are bacteriophages (bacterial viruses) which consist of a capsid containing single- or double-stranded DNA as the genome. The capsids may be of simple cubic symmetry or complex structures with heads, tails, tail-fibres etc. They belong to the morphological groups A to D and are classified into the following families: *Myoviridae* (ds DNA, long contractile tails, capsids up to 100 nm), *Siphoviridae* (ds DNA, long non-contractile tails, capsids 50 nm), *Podoviridae* (ds DNA, short non-contractile tails, capsids 50 nm) and *Microviridae* (ss DNA, no tail, capsid 30 nm). Somatic coliphages are virulent

phages which attach to lipopolysaccharide or protein receptors in the bacterial cell wall and may lyse the host cell in 20 to 30 min under optimal conditions. They produce plaques of widely different size and morphology. The presence of somatic coliphages in a water sample usually indicates pollution by human or animal faeces or by wastewaters containing these excreta (ISO 10705-2 2000). Their distribution in the digestive tract of humans and animals has been studied several times and it has been shown that 23.5% of human stool samples contain *E. coli* phages with a concentration of 10^5 PFU per gram of faeces.

Somatic coliphages are not present in Italian normative on water quality but for their size are considered a good indicator of treatment performances in filtration treatments. Somatic coliphages were quantified according to the ISO 10705-2 (2000) method: the sample is mixed with a small volume of semi-solid nutrient medium. A culture of host strain is added and plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. The results are expressed as the number of plaque (also termed plaque-forming units, pfu), per unit of sample volume. For samples with a high bacterial load, such as wastewater, the recommended indicator bacterium is the *E. coli* strain resistant to nalidixic CN (ATCC 70078), also known as WG5. The procedure provides to aseptically add 300 µL of calcium chloride solution (at room temperature) to 50 mL of ssMSA and nalidixic acid up to a final concentration of 250 µg/mL. Aliquot 2.5 mL of solution into sterile tubes with a stopper, inside a thermostat bath at 45±1 °C, in order to keep the preparation liquid. Transfer to each tube 1 mL of sample (as it is or diluted), providing more tests in parallel for each sample and for each dilution. Add 1 mL of host cell culture, mix thoroughly taking care not to create air bubbles, then pour onto Petri dishes, on which a layer of MSA has already solidified. Allow to dry and incubate at 36±2 °C for 18±2 hours. Count the lysis plates after 18-20 hours of incubation and express the result in pfu in a determined volume of sample analyzed. Select plates that have good separation of the lysing plates and have, if possible, more than 30 plates; if there were none, select the plates inoculated with the largest sample volume. Calculate the number x, which represents the number of plaque-forming units present in 1 mL of sample (eq. 7).

$$x = \frac{N}{(n_1 V_1 F_1)+(n_2 V_2 F_2)} \qquad \textbf{(Eq. 7)}$$

x = number of plaque-forming units present in 1 mL of sample (pfu/mL)
N = total number of plates counted on selected Petri dishes
n_1, n_2 = number of replicas counted for the dilutions F_1, F_2
V_1, V_2 = sample volume, expressed in mL, used for the dilutions F_1, F_2
F_1, F_2 = dilution used for the sample to be analyzed (F = 1 for the sample as such, F = 0.1 for the sample diluted ten times, etc.)

Each ten-fold serial dilution tested was analysed in quadruplicate. For SF effluent, 20 mL of undiluted sample were scrutinised.

Genes for antibiotic resistance

Detection of antibiotic resistance genes (ARGs) was performed by bio-molecular approach. This method is based on the detection of DNA/RNA sequences specific of the targeted gene or microorganism allowing its identification and/or quantification in complex environmental matrices.

All water samples were concentrated by filtration on 0.22 µm poresize polycarbonate membrane sterile filters (diameter: 47 mm, Whatman, UK). The filtrated volumes were in the range of 50–75 mL for raw influent and of 150–300 mL for secondary and tertiary effluents depending on the sample filterability. Filters and biomass samples were stored at −20°C until DNA extraction.

DNA was extracted directly from the filter membranes following a phenol:chloroform:isoamyl alcohol protocol (Miller et al., 1999) with minor modifications. In detail, a cell lysis step by enzymatic treatment was performed in addition to the bead beating approach described in Miller et al. (1999). For the cell lysis, the filters were firstly incubated at 37°C for 40 min with lysozyme (20 mg/mL), (Sigma Aldrich, USA) in lysis buffer (200 mM NaCl, 200 mM Tris, 2 mM NaCitrate, 10 mM $CaCl_2$, 50 mM EDTA, pH 8.0) and successively, incubated at 50°C for 30 min with the addition of Proteinase K (5

mg/mL), (Sigma Aldrich, USA). The quality of the extracted DNA was analyzed with Nanodrop 2000c (Thermo Scientific, Italy). DNA was stored at −20 °C before the analysis. PCR was applied to the collected samples for assessing the presence of the following nine antibiotic resistance genes: *ampC, mecA, ermB, sul1, sul2, tetA, teO, tetW, vanA*, according to previously described protocols (Aminov et al., 2001; Böckelmann et al., 2009; Heuer and Smalla, 2007; Ng et al., 2001). All PCR reactions were run on a Perkin Elmer GeneAmp 2400 Thermal Cycler PCR in 50 µL-reaction mixture containing 1X PCR buffer, 200 µM dNTPs, 0.2-0.5 µM for each primer, 1.25 U of TaqDNA Polymerase (5PRIME), and 1 µL of template DNA (dilutions from 100 to 500 ng DNA). Nuclease-free water was used as negative control for each run. The correct length of the amplified PCR product was evaluated by 1% agarose gel electrophoresis and sequenced to confirm the specificity of PCR amplification.

The four antibiotic resistance genes detected by PCR (*ermB*, *sul1*, *sul2*, *tetA*) and 16S rDNA gene were quantified by Q-PCR. In comparison with other applied molecular methods for ARGs detection, such as PCR, microarrays and southern blot, Q-PCR provide quantitative data (Rizzo et al. 2013) necessary for the evaluation of ARGs removal in treatment systems. Additionally in respect to culture based methods for monitoring antibiotic resistances Q-PCR allows the detection and quantification also of ARGs present as free extracellular DNA in the environment and thus more susceptible to induce horizontal gene transfer. However, several well know limitations of Q-PCR, such as the possible presence of Q-PCR inhibitors in the analysed samples, should be carefully considered. The presence of Q-PCR inhibitors introduces a number of problems, ranging from reduced amplification efficiency and reduced assay sensitivity to complete reaction failure.

Thus, in the current study, serial dilutions of the extracted DNA were analysed to assess qPCR inhibitions, including no template controls (NTCs) for each qPCR assays.

Q-PCR TaqMan assays were utilised for 16S rDNA (Suzuki et al., 2000), *ermB* (Bockelman et al. 2009), *sul1* (Heuer and Smalla 2007) and *sul2* (Heuer et al., 2008), while *tetA* was quantified according to Ng et al. (2001) utilising SYBR Green based Q-PCR. Primers and probes sequences as well as the applied Q-PCR conditions are reported in Table 2.5.2. Q-PCR was performed with CFX96 Touch Real Time PCR Detection System (Bio-Rad, USA) in 25 µL volume. Q-PCR reactions contained 5 µL DNA template, 12.5 µL of 2X Mastermix (SsoAdvanced™ Universal Probes Supermix (Bio-Rad, USA) for *ermB*,

sul1 and *sul2* and SYBR Green Supermix (Bio-Rad USA), primers and probes at the required concentrations (Table 2.5.2.).

NTCs with no template DNA were included in each Q-PCR assays and serial dilution of the sample DNA extract corresponding to 50, 20 and 5 ng (500, 50, and 10 ng) DNA/reaction tube were analysed to assess presence of Q-PCR inhibitions. Each reaction was run in triplicate data were analysed with the software CFX Manager.

The quantity of target gene in unknown samples was calculated on the base of a standard curve (Ct value versus log of initial gene copy number) obtained using known quantities of the plasmid DNA carrying target genes (i.e. *ermB, sul1, sul2, tetA*). Plasmid positive controls for *sul1* and *sul2* were purchased from DSMZ plasmids, R388 (DSMZ 5189) and RSF1010 (DSMZ 5401) respectively. Positive controls for ermB and tetA were instead obtained by cloning PCR products ligated into pGEM®-T Easy plasmid. For standards production, plasmid DNA was extracted using the according to the QIAprep™ Spin Miniprep Kit (QIAGEN). The concentration of the purified plasmid DNA was determined using NanoDrop spectrophotometer. The copy number of ARG and 16S rDNA gene per µl of plasmid solution was then calculated as described in Czekalski et al. (2012). Ten-fold serial dilutions of plasmid DNA (gene copy numbers from 10^2 to 10^6 per reaction) were amplified in triplicate with the unknown samples for each qPCR assay.

ARGs	Primer and probe sequences (5'-3')	Amplicon size (bp)	Annealing/ extension temperature (°C)	References
tetA	F:GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	210	55	Ng et al. 2001
ermB	F: GGATTCTACAAGCGTACCTTGGA R: GCTGGCAGCTTAAGCAATTGCT P:FAM-CACTAGGGTTGCTCTTGCACAC-TCAAGTC–BHQ-1	92	60	Bockelmann et al. 2009
sul1	F: CCGTTGGCCTTCCTGTAAAG R: TTGCCGATCGCGTGAAGT P:FAM-CAGCGAGCCTTGCGGCGG-TAMRA	67	60	Heuer and Smalla et al. 2007
sul2	F: CGGCTGCGCTTCGATT R: CGCGCGCAGAAAGGATT P:FAM-CGGTGCTTCTGTCTGTTTCGCGC-TAMRA	60	60	Heuer et al. 2008
16S rDNA	F: CGGTGAATACGTTCYCGG R: GGWTACCTTGTTACGACT P: FAM-CTTGTACACACCGCCCGTC-TAMRA	124	56	Suzuki et al. (2000)

Tab. 2.5.2. Primers, probes and thermal cycling conditions for Q-PCR.

2.5.5. Sludge production

When a fixed value of headloss was reached (3 bar) at the bottom of the biofilter a washing step was carried out by means of compressed air until the headloss decreased to a predefined value (1 bar). The washing water was collected and Total and Volatile Suspended Solids, (TSS and VSS, respectively) were measured in order to calculate the specific sludge production. The frequeny amd intensity of washing operations are an important operative parameters for SBBGR systems since they influence sludge retention time and concentration whitin the biofilter and consequently the quantity of sludge produced. In fact, sludge can leave the SBBGR system either with the effluent (i.e., as suspended solids) or as a result of a washing operation. Unlike first way, the amount of sludge expelled by washing operation can be controlled by changing the operative parameters of the operation (e.g., by increasing/decreasing the set point value for carrying out the washing; by changing the headloss drop during the washing; etc) (Di Iaconi et al. 2010).
The specific sludge production was calculated dividing the amount of sludge expelled from the SBBGR plant (TSS removed with the effluent + TSS removed during the wash operations) by the amount of COD removed during experimentation time (eq. 8.).

$$\text{Specific sludge production} = \frac{TSS_{eff} + TSS_{wash\ operation}}{COD_{inf} - COD_{eff}} \qquad \textbf{(Eq. 8.)}$$

2.6 RESULTS AND DISCUSSION

2.6.1. Treatment performance evaluation in terms of physical and chemical parameter removal

The treatment performance of the SBBGR implant was evaluated by periodic analysis of influent and effluent samples recorded during eight months of operation. Table 2.6.1. reports influent and effluent concentrations of the main gross parameters with their relative removal efficiency.

The values shown that the performance of the pilot plant in terms of COD and BOD_5 removal turned out to be excellent. In fact, an average removal efficiency of 92% for the COD and 99% for the BOD_5 was recorded. The total output COD value has always been below the 100 mg/L limit set by italian legislation, moreover, BOD_5 in the biologically treated wastewater is on average 1 mg/L, always much lower than the quality levels required in Italy for reuse in agriculture (less than 20 mg/L).

Examining the table 2.6.1., it can be seen that the SBBGR system has always produced an effluent with a low solids content. In fact, TSS removal efficiency was on average 98% with residual concentration in the effluent on average 4 mg/L, meeting the reuse quality criteria of the Italian legislation (limit of 10 mg/L). These excellent performance of removal of solids and organic matter are due to the characteristics of the SBBGR system. In the biofilter the biomass growing dense and compact acts as a filter to retain the particulate, then the organic matter trapped between the biomass is degraded by microorganisms and used as a substrate.

The examination of the values of TKN and ammonia entering the plant shows, according to the type of waste treated, that the TKN consists mainly of ammonia. Regarding the removal of nitrogen, it is verified that the plant is able to remove on average 99.8% of ammonia, this capacity is confirmed by the TKN output values on average of 3.8 mg/L. The removal efficiency recorded for total nitrogen (on average 78.2%) demonstrates that in addition to a complete nitrification process there is also a denitrification process that leads to the production of gaseous nitrogen. This ability of the system is ascribed to the high value of biomass concentration present in the reactor bed, which allows the cohabitation

of nitrifying and denitrifying bacteria in the biomass layers as reported in De Sanctis et al. (2010).

The phosphorus removal in a biological system is linked to microbial growth and therefore to the production of sludge, low phosphorus removal is to be expected. Table 2.6.1, show instead a rather high average removal efficiency, equal to 38.7%.These high phosphorus removal values are ascribable to the filtering capacity of the SBBGR system, and therefore to the ability to retain suspended solids containing phosphorus.

Parameter		Mean value ± S.D.
COD	Influent [mg/L]	508 ± 226
	Effluent [mg/L]	40 ± 16
	Removal efficiency [%]	92 ± 4
BOD_5	Influent [mg/L]	285 ± 86
	Effluent [mg/L]	1 ± 2
	Removal efficiency [%]	99 ± 1
TSS	Influent [mg/L]	246 ± 115
	Effluent [mg/L]	4 ± 2
	Removal efficiency [%]	98 ± 1
NH_3	Influent [mgN/L]	53.8 ± 25.5
	Effluent [mgN/L]	0.1 ± 0.2
	Removal efficiency [%]	99.8 ± 0.6
TKN	Influent [mg/L]	75.3 ± 26.8
	Effluent [mg/L]	3.8 ± 2.5
	Removal efficiency [%]	95.9 ± 2.6
TN	Influent [mg/L]	75.4 ± 26.7
	Effluent [mg/L]	14.2 ± 6.9
	Removal efficiency [%]	78.2 ± 14.2
P-tot	Influent [mg/L]	8.1 ± 4.3
	Effluent [mg/L]	3.7 ± 0.6
	Removal efficiency [%]	38.7± 32.1

Table 2.6.1. Performances of biological treatment by the SBBGR system .

The treatment performance of the SBBGR system monitored for a period of 230 days (once a week) is shown below in more detail. In this period the system operated in stable operating conditions (table 2.6.2.) with an organic loading rate of 1.2 ± 0.5 KgCOD/m^3 $_{bio-filter}$ d, comparable to that of conventional systems. In the same period and with the same operating conditions, disinfection tests and microbiological tests, subsequently reported, were carried out.

SBBGR	
Volume (biofilter + aerator) (L)	300
Wastewater recirculation (L/h)	120
Wastewater up-flow velocity into biofilter (m/h)	3
Aeration (m^3/h)	3.5
Cycle length (h)	6
Hydraulic loading rate (L/d)	240
Hydraulic retention time (h)	30
Organic loading rate (KgCOD/m^3 $_{biofilter}$ d)	1.2± 0.5

Tab. 2.6.2. Main features and operating conditions of SBBGR

pH, EC, SAR.

In case of agricultural reuse of the treated wastewater is important monitor parameters like pH, EC and SAR. Figure 2.6.1 shows average values of pH, EC and SAR measured in the influent and effluent of SBBGR.

The pH values in the effluent of the SBBGR are on average 7.8 and fall within the range of values between 6.5 and 8.5 required for agricultural reuse. Italy and other countries have established limit values also for EC and SAR (Paranychianakis et al., 2015). High values of these parameters in water used for irrigation can cause changes in the structure and properties of the soil, damaging plants and soil microorganisms. The high absorption capability of SBBGR biomass allows to significantly reduce EC value from 1.22 (average influent value) to 0.88 mS/cm (average effluent value). Italy has set the maximum values of 3 mS/cm and 10 for EC and SAR respectively. SBBGR effluent has EC and SAR values always lower than 1 mS/cm and 2.8 respectively, which is why it is suitable for crop irrigation.

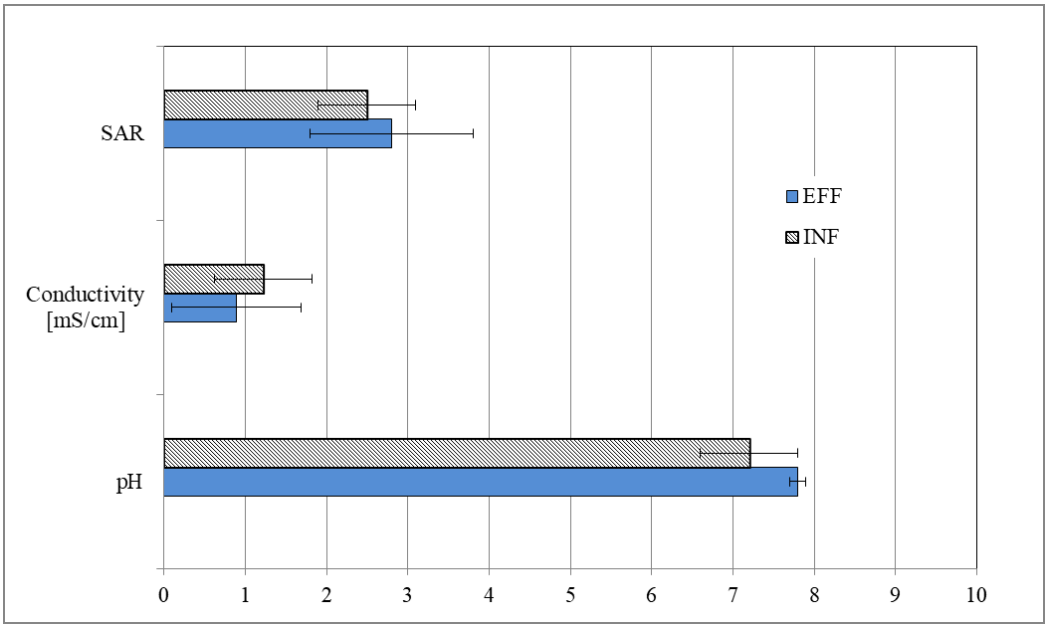

Fig. 2.6.1. pH, EC and SAR in influent and SBBGR effluent.

Organic matter.

The examination of the concentration profiles of the total and soluble COD entering the plant, shown in figure 2.6.2., indicates a particular feature of the treated wastewater (wastewater from a small community) that is to have a high ratio between the total COD and the soluble one. This derives from the limited extension of the sewage system which makes the process of hydrolysis of the particulate substrate no-existent or very limited. Therefore, the organic content of these wastewater is present mainly in particulate form. For the wastewater treated in the present study, the COD_{total} /$COD_{soluble}$ ratio is on average of 2.5. Observing figure 2.6.3. is highlighted a good ability to reduce COD during the entire monitoring period (about 8 months). The efficiency of removal is maintained at around 92%, and has never fallen below 75%, a value imposed by the Italian legislation (Legislative Decree 152/2006). It should also be noted that such high removal efficiencies have also been obtained with a large variability in the input value.

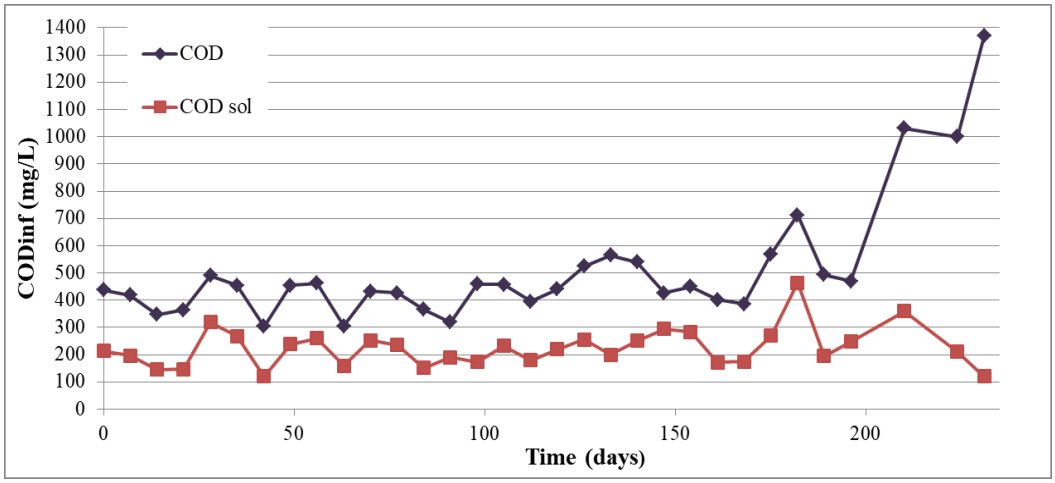

Fig. 2.6.2. Profiles of total and soluble COD values of SBBGR influent. The data shown are referred to the monitoring period: zero represents the first day of analysis.

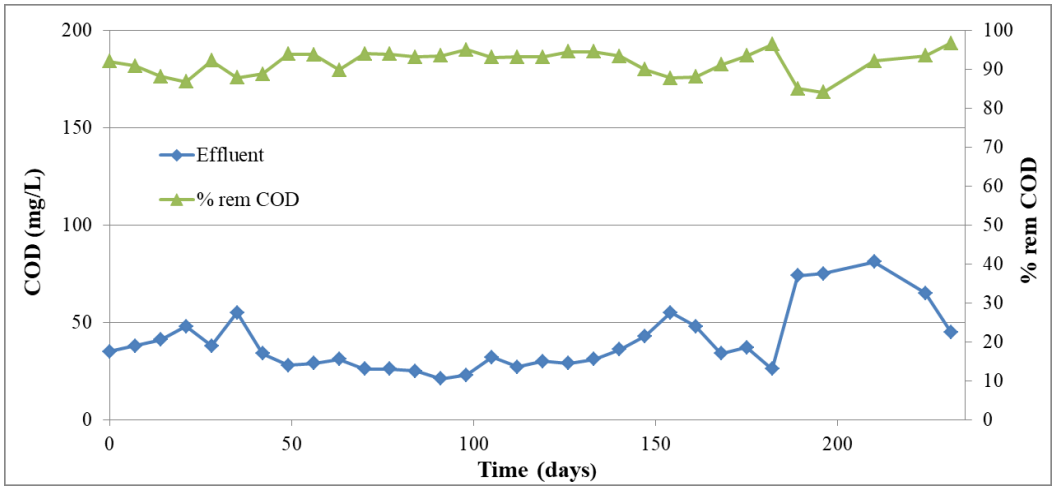

Fig. 2.6.3. Profiles of the input and output values and the related removal percentage for the COD parameter. The data shown are referred to the monitoring period: zero represents the first day of analysis.

In fact, the value of the input COD fluctuated between the maximum value of 1370 mg/L (recorded on day 231) and the minimum value of 304 mg/L of day 42 (see figure 2.6.2). The efficiency of the COD removal in these two days (borderline cases) was nevertheless always high (i.e., 97% and 89%). This is due to the high stability and flexibility of the

SBBGR system, which thanks to its particular type of biomass (mixture of granules and biofilm confined in a filling medium) is able to adapt to fluctuations operating conditions. The value of the residual COD in the effluent is on average 40 mg/L and in any case never exceed 100 mg/L, limit imposed by the Italian legislature for agricultural reuse, in fact the presence of biodegradable COD, favoring the development of biofilm, could obstruct and damage water diffusers.

Solids

In case of agricultural reuse of water treated, COD and TSS parameters acquire greater relevance and a functional as well as qualitative value. High concentrations of TSS in the effluent may cause the irrigation network clogging, especially in drip irrigation systems. The $VSS_{influent}/TSS_{influent}$, close to the unit (figure 2.6.4.) indicates that the total suspended solids in the influent are made up almost of organic matter, confirming the above mentioned for the COD parameter.

Figure 2.6.5. shows the concentration profiles of the total suspended solids leaving the plant and their removal efficiency. From the examination of figure 2.6.5, it can be observed that the SBBGR system has always produced an effluent with a low solids content, the concentration of total suspended solids in the effluent was found to be often below 10 mg/L, limit set by national legislation for crops irrigation. Furthermore, sand filtration allowed to further reduce suspended solids concentration constantly below 5 mg/L.

The values show excellent performance of the pilot plant in terms of removal of suspended solids. In fact, an average removal efficiency of 98% with peaks of 99% was recorded, even with a strong fluctuation of the input value (between 133 and 634 mg/L, see figure 2.6.4).

Nitrogen compound

The examination of TKN and ammonia profiles (figures 2.6.6. and 2.6.7.) in SBBGR influent shows that TKN consists mainly of ammonia. In fact, the nitrogen is present for 60-70% as inorganic nitrogen that the plant is able to completely remove. Figure 2.6.6. shows a removal efficiency of TKN almost greater than 90% (except for a single day, when a removal efficiency of 87% was measured) with residual concentrations in the effluent on average lower than 4 mg/L, regardless of the input concentration.

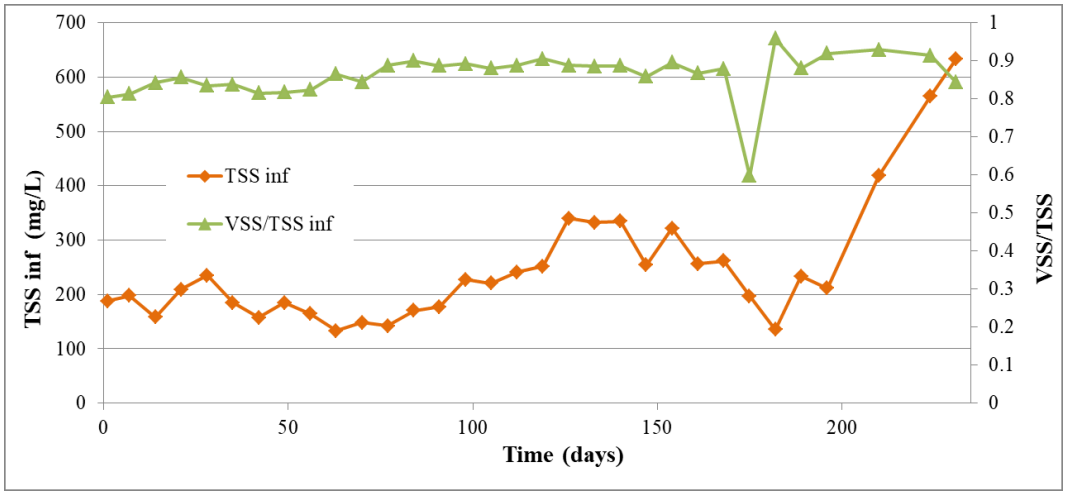

Figure 2.6.4. Concentration profiles of total (TSS) suspended solids in SBBGR influent and (volatiles) VSS/TSS ratio. The data shown are referred to the monitoring period: zero represents the first day of analysis

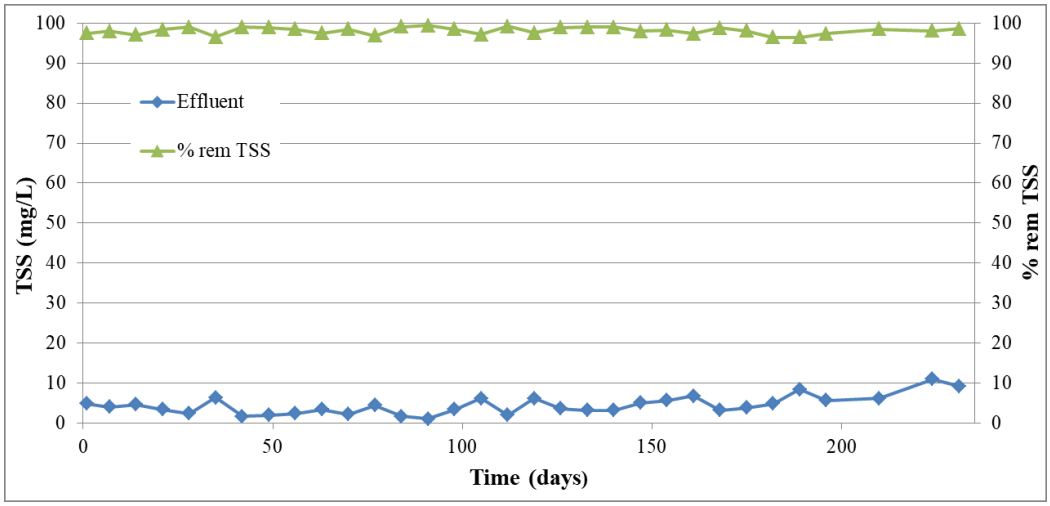

Figure 2.6.5. Total suspended solids (TSS) concentration profiles in SBBGR effluent and relative removal efficiency. The data shown are referred to the monitoring period: zero represents the first day of analysis.

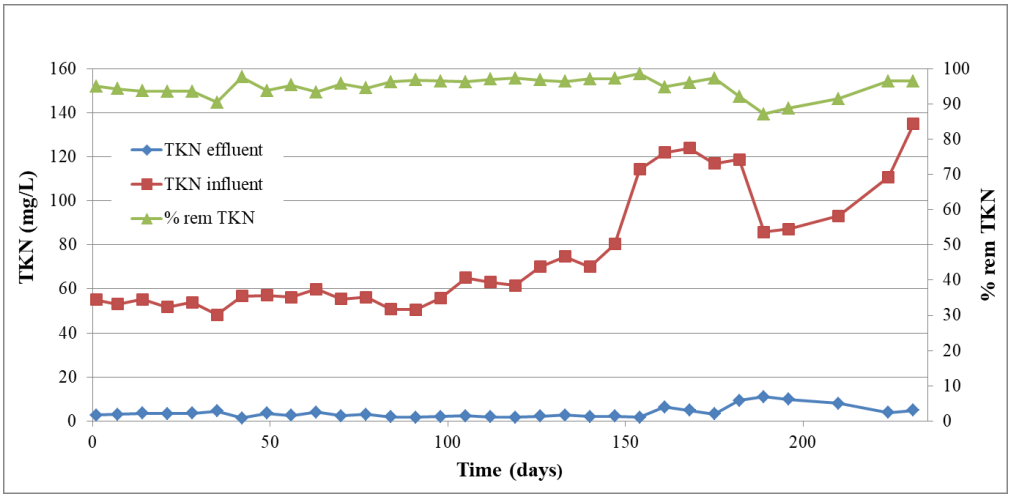

Fig. 2.6.6. Profiles of the input and output values and the related removal percentage for the TKN parameter. The data shown are referred to the monitoring period: zero represents the first day of analysis.

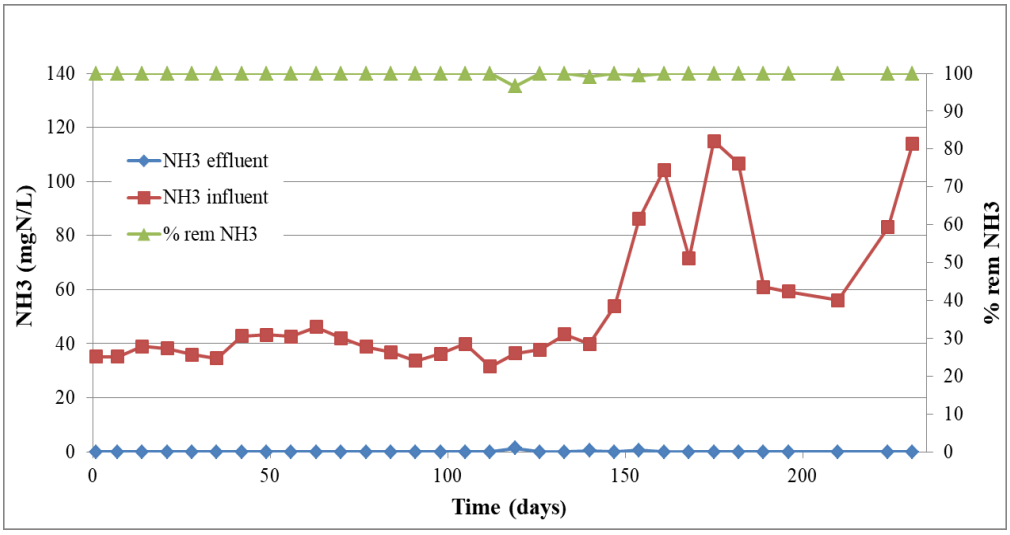

Fig. 2.6.7. Profiles of the input and output values and the related removal percentage for the ammonia parameter. The data shown are referred to the monitoring period: zero represents the first day of analysis.

The results obtained for the ammonia parameter were even better than those for the TKN. In fact, the profiles shown in figure 2.6.7 underline that the plant has always produced an ammonia-free effluent, regardless of the concentration value in the influent that has fluctuated between 31.5 and 115 mg/L.The high removal efficiency of TKN and ammonia can be attributed to the existence of a nitrification process (i.e., ammonia oxidation, to nitrite and then to nitrate) stable as confirmed by mean removal values and standard deviation of TKN and ammonia of 94.9 ± 2.6 % and 99.8 ± 0.6% respectively. The existence of the nitrification process is confirmed by the profiles of nitrous nitrogen and nitric nitrogen, reported respectively in figure 2.6.8. and 2.6.9. Moreover, from the profiles of nitrous nitrogen in the effluent it can be observed how the concentrations of the oxidized nitrogen have always been lower than 0.5 mg/L except for two values (0.6 and 2.5 mg/L) and that, therefore, the nitrification process reaches up to nitrate whose concentrations in the effluent have values between 1.2 and 25.8 mg/L.

The total nitrogen profiles (TN) shown in figure 2.6.10. demonstrate the existence of a rather extensive denitrification process (i.e. reduction of oxidized nitrogen to gaseous nitrogen), although the treatment cycle performed by the plant did not include a final programmed anoxic phase (i.e. a phase during which no air is supplied). Denitrification is a form of anaerobic respiration that uses nitrate (or nitrite) as the final acceptor of electrons in the absence of oxygen. The TN output concentration values have always been less than those in input; the missing quantity is presumably formed from nitrogen necessary for microorganisms as a nutrient and molecular gaseous nitrogen which leaves the liquid phase to reach the atmosphere. The simultaneous nitrification-denitrification process can be attributed to the high concentration of biomass usually present in the biofilter unit of the SBBGR system (higher than 40-50 kgTSS/m^3) and to the dynamic operating conditions (typical of sequential reactors) which generate inside the bed, adjacent aerobic and anoxic zones (where nitrification takes place in the first, and denitrification in the second).

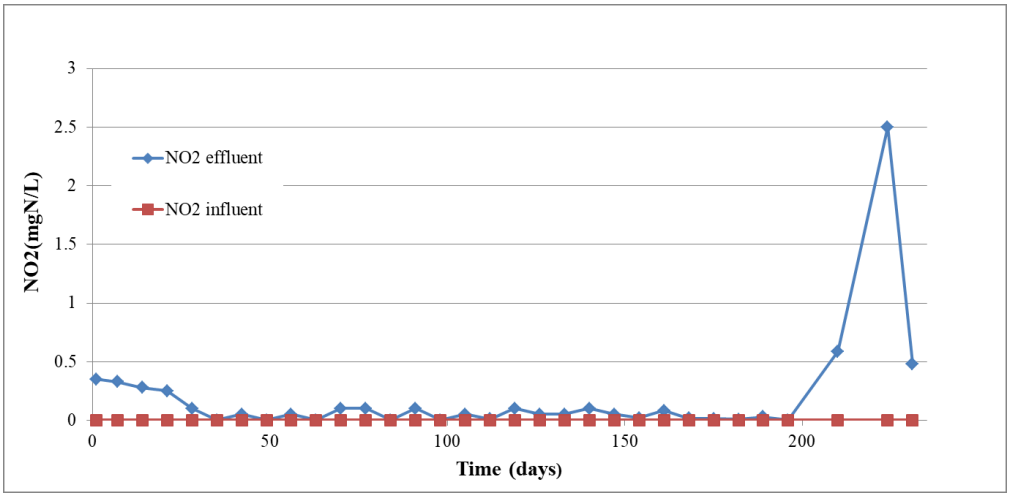

Fig. 2.6.8. Profiles of the input and output values of nitrous nitrogen. The data shown are referred to the monitoring period: zero represents the first day of analysis.

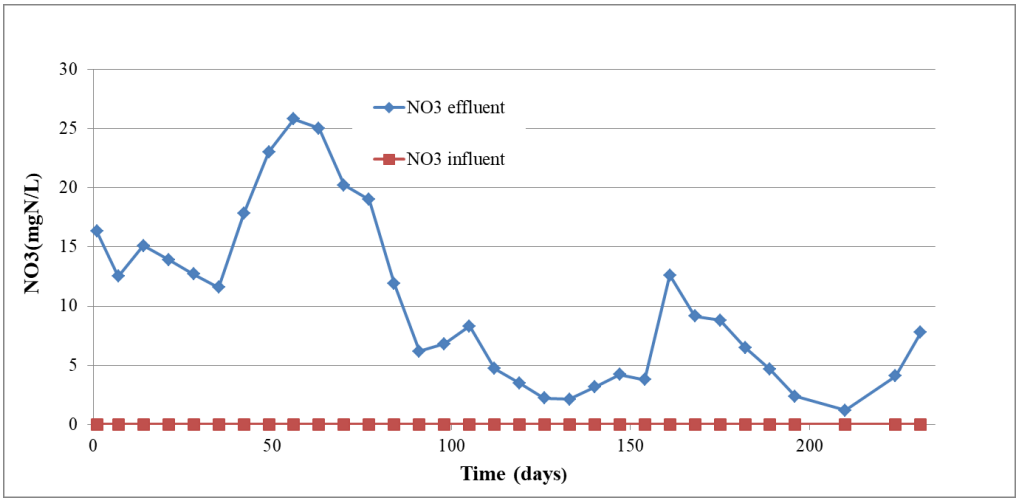

Fig. 2.6.9. Profiles of the input and output values and the nitric nitrogen. The data shown are referred to the monitoring period: zero represents the first day of analysis.

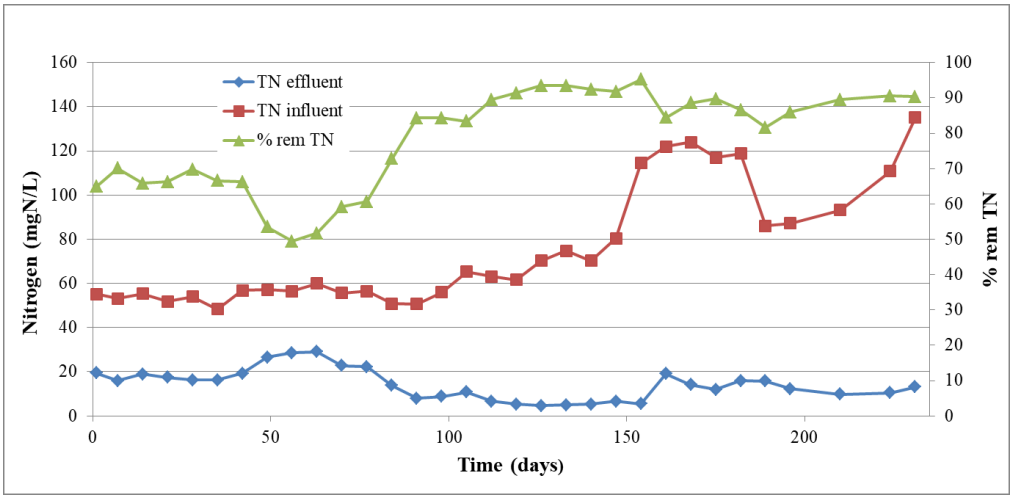

Fig. 2.6.10. Profiles of the input and output values of total nitrogen and the related removal percentage. The data shown are referred to the monitoring period: zero represents the first day of analysis.

Figure 2.6.10. shows values of removal efficiency of the TN on average of 78%. The values are variable, from 50 to 95%, due to the COD/N ratio which sometimes turned out to be rather low (less than 6), thus indicating an organic carbon deficiency for complete denitrification of oxidized nitrogen (in such circumstances, an external carbon source could to be provided in order to improve nitrogen removal).
The average values of ammonia obtained in the effluent, respect the limits established by the Italian legislation on the reuse of the effluents purified for irrigation (2 mg/L). Regarding total nitrogen values, although the limit imposed by the D.M. 185/03 for the parameter total nitrogen is equal to 15 mgN/L, in agriculture this element, if well dosed, is necessary for the fertilization of crops, then the local authorizaties are delegated by the D.M. to raise the limit to 35 mg N/L, without prejudice to the provisions of art. 10, paragraph 1, concerning the areas vulnerable to nitrates of agricultural origin.

Phosphorus

Phosphorus, together with nitrogen, is one of the essential components for the growth of microorganisms; the phosphorus content of a conventional biomass is about 1-2% of its weight (in terms of VSS). Phosphorus removal in a biological system is therefore linked to

microbial growth and thus to sludge production. For SBBGR systems, which are characterized by low sludge production, low phosphorus removal is to be expected. The total phosphorus concentration profiles in and out of the plant (figure 2.6.11), show instead a rather high average removal efficiency, equal to 38% with a residual effluent concentration on average less than 4 mg/L. This result would suggest the presence of a biological phosphorus removal from organisms that accumulate phosphorus (PAO) (De Sanctis et al. 2017) even if these microorganisms require a specific sequence of environmental conditions in the reactor (i.e., an anaerobic phase followed by an aerobic one: Adav et al., 2008). Considering that there is no planned anaerobic phase in SBBGR system, the presence of a chemical or physical phosphorus removal processes is probable. For example, due to the very high concentration of biomass in the biofilter, phosphorous present in the form of particulate can be retained by biomass.

The limit imposed for this parameter by the D.M. 185/03 is equal to 2 mg P/L. However, this element is necessary for the fertilization of crops, which is why for the agricultural reuse, the local authorizaties are delegated by the D.M. to raise this limit to 10 mgP/L, limit always respected during the experimentation period, as shown in figure 2.6.11.

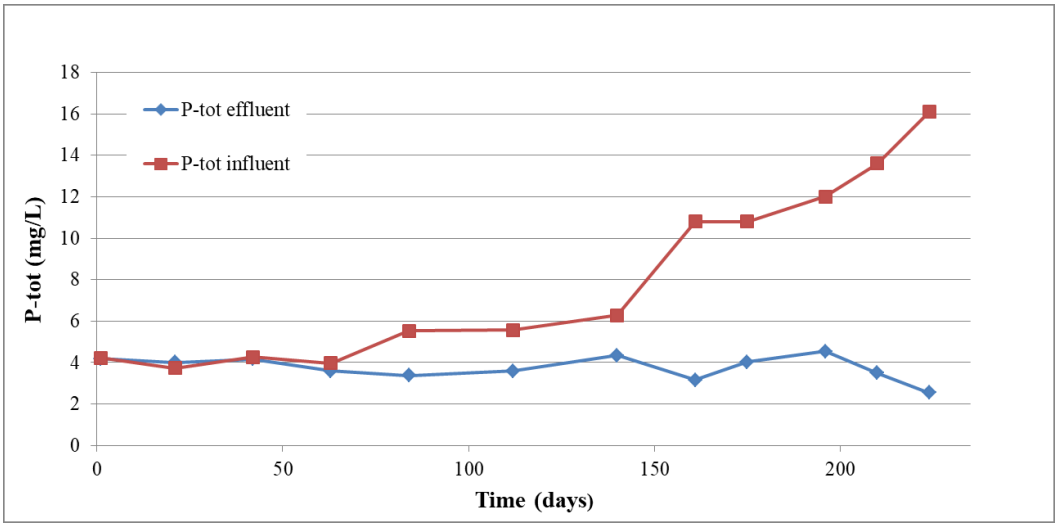

Fig. 2.6.11. Profiles of the input and output values of total phosphorus. The data shown are referred to the monitoring period: zero represents the first day of analysis.

According to the monitored “gross parameters” the quality of SBBGR effluent allows its agricultural reuse in Italy and in several European countries (Paranychianakis et al., 2015)

Sludge production.

During the investigated period washing operations were performed, as the setpoint value of 3 bar was reached. According to what reported in the materials and methods, the specific sludge production was calculated by dividing the amount of sludge expelled from the SBBGR plant (SST removed with the effluent + SST removed during the washing operations) for the quantity of COD removed during the two periods.

The balance of the expelled sludge and the COD removed during this time gave a sludge production value of about 0.12-0.14 kgTSS/kg$COD_{removed}$. This value is much lower (up to 80%) than that of conventional wastewater treatment systems based on activated sludge systems (Schultz et al., 1982). This result turns out to be of great interest both from the management point of view of the plant and from the economic point of view.

In Europe sludge production is continuously increasing (from 5.5 million tons of dried sludge in 1992 to 8 million in 1998 and 10 million in 2007), furthermore the disposal has estimated costs between € 350-750 per tonne of dried sludge (Foladori et al., 2010; Ginestet, 2007).

The process was characterised by a very low sludge production ascribable to the high age (>120 d) and concentration (25-30 gVSS/L_{bed}) of the biomass in the system.

Biomass in fact, besides being constituted also by nitrifying bacteria characterized by a low growth rate, in the operating conditions described spend much time in the endogenous metabolism phase, with a sharp reduction in the growth rate.

This can be also confirmed by the low VSS/TSS ratio (about 0.6) of excess sludge collected after washing operation. Such good level of stabilization of excess sludge also makes it possible to avoid a further sludge stabilization process before sludge drying and final disposal.

2.6.2. Treatment performance evaluation in terms of microbial indicators and pathogens

The disinfection efficiency of the proposed treatment was evaluated monitoring the following microbial indicators and pathogens after each treatment phase: *Escherichia coli* and *Salmonella* (representative of bacteria), *Clostridium perfringens* spores (representative of spore-forming bacteria), somatic coliphages, human enteroviruses and adenoviruses (representative of viruses), *Giardia* and *Cryptosporidium* (rapresentative of protozoa). Furthermore, the presence of four different genes, identified as antibiotic resistance genes, was evaluated.

The effectiveness of the disinfection process was assessed by measuring the concentration of the selected microbiological indicators, before and after each treatment step, and calculating the removal efficiency. The removal efficiencies were expressed in terms of log units removed (LUR) according to the following equation:

$$LUR = \log c_a/c_b$$

where c_a and c_b are the concentrations of the specific microbial indicator after and before the specific treatment.

The removal efficiency was calculated for effluent samples of the SBBGR treatment only, for effluent samples after sand filtration and for effluent samples after filtration and disinfection treatment (UV or peracetic acid). As it is known, in fact, the performances of a disinfection system through UV rays are influenced by the presence of suspended solids in the effluent; despite the effluent of the SBBGR system is characterized by a low concentration of suspended solids, it was still preferred to insert a sand filtration step (treatment, however, always advised, due to its low cost) upstream of UV disinfection, in order to maximize the efficiency of the UV lamp and increase the specificity action of tertiary treatment.

The disinfection performances of the biological treatment by SBBGR and sand filtration are reported for each parameter investigated in the tables from 2.6.3. to 2.6.8.

Table 2.6.3 shows how the biological treatment with the SBBGR system is able to remove 2.5 units of total coliforms. This result is of particular interest when compared to the performance obtained from plants based on conventional technologies reported in the literature. Koivunen et al. (2003) report a total removal of the coliforms between 2 and 3 units of logs, but this value refers to the effluent of four Finnish treatment plants after prelimi-

nary, primary and secondary phases. Furthermore, Zhang et al. (2007) report a total coliforms removal on average only of 1.9 logs after secondary treatment and reaches the value of 2.5 LUR just after tertiary treatment with rotating biological contactor, sand filtration and chlorine contactor. Therefore, these results show clearly that SBBGR is able to offer, in a single step, almost the same removal of total coliforms obtained by conventional plants through several steps. Sand filtration further improves the removal efficiency of the SBBGR system up to 3.6 LUR.

		Wastewater	**SBBGR effluent**	**SF effluent**
Total coliforms	Concentration (MPN/100ml)	$1.2 \pm 3.2 \times 10^7$	$0.8 \pm 2.4 \times 10^5$	$1.2 \pm 2.6 \times 10^3$
	LUR		2.5 ± 0.8	3.6 ± 1.3

Tab. 2.6.3. Performances of the proposed treatment in terms of concentrations of total coliforms measured in wastewater, SBBGR effluent and after sand filtration (SF effluent) (10 samples) and removal efficiencies.

SBBGR performed even better against *E. coli*, allowing removal on average of 3.8 log units and leading to an average concentration in the effluent of $0.9 \cdot 10^3$ MPN/100 mL (table 2.6.4). Several studies have reported concentrations of *E.Coli* much higher, in the renge of 10^4-10^5 MPN/100 mL in effluents of Italian plants based on activated sludge technology (De Luca et al., 2013; Carducci et al., 2008), the effluent of the SBBGR system is therefore of quality 10-100 times better than that of the conventional systems.
The concentration found in the effluent of the SBBGR system is lower than the limit set by Italian legislation for the discharge into surface waters (i.e. 5000 UFC/100mL) which is usually achieved with a disinfection stage. Therefore, if the SBBGR system were applied in place of the current activated sludge systems, there would no longer needed a final disinfection stage. The quality of SBBGR effluent complies with that indicated by the WHO guidelines for agricultural reuse (10^3 CFU/100 mL; World Health Organization – WHO, 2006) and further increased after the sand filtration step. In fact, the sand filter removed on average 1 log unit of *E. coli*, thus leading to an overall disinfection efficiency higher

than 4 log units, but the final concentration of *E. Coli* does not comply with the very strict limit set by Italian legislation for reuse in agriculture (10 CFU/100 mL) therefore it is necessary a disinfection treatment of the effluent, which however will certainly be much less "energetic" than that usually performed for the irrigation reuse of the effluent of conventional treatment, due to the higher quality of the SBBGR system effluent.

		Wastewater	SBBGR effluent	SF effluent
E. coli	Concentration (MPN/100ml)	$1 \pm 2.7 \times 10^7$	$0.9 \pm 2.1 \times 10^3$	$6 \pm 9.1 \times 10$
	LUR		3.2 ± 1.1	4.2 ± 1.5

Tab. 2.6.4. Performances of the proposed treatment in terms of concentrations of *E.coli* measured in wastewater, SBBGR effluent and after sand filtration (SF effluent) (10 samples) and removal efficiencies.

Another important parameter taken into consideration in case of agricultural reuse is represented by *Salmonella*. Many regulations require the complete absence of this bacterium in treated water. For this reason a presence/absence approach was performed on the influent and effluent samples. Salmonella has been detected only in some samples of raw wastewater, probably because the wastewater came from a small community and the presence of the pathogen in all the samples was not guaranteed.
Salmonella has not been detected in any effluent sample treated by the SBBGR system, respecting the Italian regulations for irrigation water.
Due to its ability to generate spores in the presence of adverse growing conditions, *C. perfringens* is extremely resistant to biological, chemical and physical disinfection strategies. The data reported in table 2.6.5. shows that SBBGR is able to ensure a stable removal of about 1 log unit of *C. perfringens*. The difficulty to remove *C. perfringens* spores is confirmed by Wen et al. (2009), this study reports a reduction of 0.98 logs for a WWTPs in Bolivar and 1.27 logs for three similar laboratory-scale plants. Lucena et al. (2004) shows that WWTP in Argentina, Spain and France, which includes primary sedimentation and activated sludge digestion, are able to obtain 1 LUR for this sporigene microorgan-

isms. The spore forming habit of sulphite-reducing *C. perfringens* gives them high environmental resistance which is considered to be significantly longer than enteric pathogens (Bisson and Cabelli, 1979). SBBGR showed removal efficiencies of this bacteria comparable to than those of conventional WWTPs, but it is important to note that the efficiency of removal more than doubles simply after sand filtration. In fact, an overall removal of 2.7 log units was obtained with a residual average content of $1.8 \cdot 10^3$ CFU/100 mL (Table 2.6.5.).

		Wastewater	**SBBGR effluent**	**SF effluent**
Clostridium perfringens	Concentration (CFU/100ml)	$5.2 \pm 5.2 \times 10^5$	$4.9 \pm 6.5 \times 10^4$	$1.8 \pm 2.1 \times 10^3$
	LUR		1.1 ± 0.4	2.7 ± 0.8

Tab. 2.6.5. Performances of the proposed treatment in terms of concentrations of *Clostridium perfringens* measured in wastewater, SBBGR effluent and after sand filtration (SF effluent) (10 samples) and removal efficiencies.

In case of reuse in agriculture, the presence of viruses must be evaluated for the protection of public health. From the Italian legislation there are no indications for the search of viruses as an index of biological contamination, however they represent a potential danger to health. Viruses are very stable in the environment and resistant to disinfection treatments practiced in wastewater treatment plant (Rodríguez-Lazaro et al., 2012). In this study the presence of somatic coliphages, adenovirus and enterovirus was investigated. Adenovirus and enterovirus were chosen as they are human pathogens, somatic coliphages were chosen because, even if they are viruses pathogenic for bacteria but not for humans, are considered a suitable indicator of virus fate in treatment processes, and are widespread in municipal and domestic wastewaters. The data in table 2.3.6. show that coliphages were present in high concentrations in raw wastewater and that were removed up to concentrations of $1.5 \pm 1.7 \times 10^4$ from the SBBGR and of $2.9 \pm 4.7 \times 10^2$ from the sand filter.

		Wastewater	SBBGR effluent	SF effluent
Somatic coliphages	Concentration (PFU/100ml)	$2.8 \pm 3.3 \times 10^5$	$1.5 \pm 1.7 \times 10^4$	$2.9 \pm 4.7 \times 10^2$
	LUR		1.4 ± 0.3	3.2 ± 0.4

Tab. 2.6.6. Performances of the proposed treatment in terms of concentrations of Somatic coliphages measured in wastewater, SBBGR effluent and after sand filtration (SF effluent) (10 samples) and removal efficiencies.

Ottoson et al. (2005) found that secondary treatment mean reductions from multiple WWTPs in Sweden were 1.04 logs for somatic coliphage, similar values (1.1 logs) after secondary treatment are also shown by Lodder and de Roda Husman (2005) (EPA 2015), Lucena et al. (2004) have reported removals of 1.5 log units of coliphages for four conventional WWTPs located in Argentina, Colombia, France and Spain.

All these results confirm the colifages treatment resistance, the removal values obtained with the SBBGR system are in line with what reported in the literature, furthermore once again the sand filtration reveals a simple and economic strategy bringing the removal efficiency of the treatment line to 3.22 logarithmic units. Unlike what reported for the somatic coliphages, enterovirus and adenovirus were detected only in two samples of raw wastewater and never in the SBBGR effluent. However, the small number of positive influent samples did not allow a significant analysis of the removal process.

Several studies on municipal wastewaters (Sedmak et al., 2005; Ottoson et al., 2006; Jebri et al., 2012) report that these viruses were present only in a small fraction of the collected wastewater samples (about 10–30%); probably being wastewater used in this study, from a small community the presence of pathogenic viruses was not guaranteed.

The SBBGR was highly efficient in the removal of *Cryptosporidium* and *Giardia.* The average concentration of *Cryptosporidium* in raw wastewater was 4.7×10 oocysts/L the SBBGR treatment ensured its almost complete removal. In fact, an average removal of 1.8 log units was obtained (table 2.6.7), and it was found only in 30% of the effluent samples with a concentration of about 1 oocyst/L.

Giardia was present in higher concentrations in the waste to be treated ($1.3 \pm 1.6 \times 10^3$), for this its presence has been detected in all the effluent samples. However, the SBBGR system showed a good ability to remove this protozoa of about 1.5 LUR (table 2.6.8).

		Wastewater	SBBGR effluent	SF effluent
***Cryptosporidium* oocysts**	Concentration (oocysts/L)	$4.7 \pm 4.7 \times 10$	0.6 ± 0.5	ND
	LUR		1.8 ± 0.3	> 2.4 *

*The log units removed were calculated considering the detection limit

Tab. 2.6.7. Performances of the proposed treatment in terms of concentrations of *Cryptosporidium* oocysts measured in wastewater, SBBGR effluent and after sand filtration (SF effluent) (10 samples) and removal efficiencies.

Castro-Hermida et al. (2008) carried out a study on twelve Spanish municipal WWTPs to investigate the removal of *Crytosporidium* and *Giardia*. Eight plants included primary and secondary treatments (based on activated sludge systems), three also included a tertiary treatment by UV irradiation and one was based on biological aerated filter technology. The authors observed that all plants were ineffective against both protozoa, leading to a removal of less than 1 log unit of *Cryptosporidium* and *Giardia*. A study on four Italian municipal WWTPs reports that the removal of *Giardia* is in the range of 1-2 log units, the highest removal efficiency is reached only after filtration and chemical disinfection of the secondary effluent (Cacciò et al. 2003).

The cited results indicate that tertiary disinfection processes are necessary in addition to the conventional active sludge process to achieve the removal of this pathogenic protozoa. In contrast, the biological treatment by SBBGR was able in a single step to ensure for both protozoa a removal higher than that reported for conventional WWTPs. The best performance of the SBBGR plant is due to the ability of the biomass to retain cysts and oocysty and subsequently degrade them, this is not possible in conventional plants where cysts and oocysts do not sediment effectively and are discharged with the effluent.

Concentrations of *Cryptosporidium* and *Giardia* in plant effluent further decreased after sand filtration treatment. In particular, *Cryptosporidium* was never detected in SF effluent, whereas concentrations lower than 2 cysts/L of *Giardia* were detected.

		Wastewater	SBBGR effluent	SF effluent
***Giardia* cysts**	Concentration (cysts/L)	$1.3 \pm 1.6 \times 10^3$	$2.9 \pm 3.6 \times 10$	0.7 ± 0.7
	LUR		1.5 ± 0.9	2.9 ± 0.6

Tab. 2.6.8. Performances of the proposed treatment in terms of concentrations of *Giardia* cysts measured in wastewater, SBBGR effluent and after sand filtration (SF effluent) (10 samples) and removal efficiencies.

In order to further reduce the presence of the investigated faecal contamination and pathogenic indicators, to reach the quality levels required by Italian legislation for reuse, the biological treatment was enhanced by combining the SBBGR treatment with chemical (by peracetic acid) and physical (by UV radiation) disinfection process. On the basis of the high quality of the SBBGR effluent, a UV radiation dose of 40 mJ/cm^2 and 1 mg/L of PAA were tested. Such low doses allow an energy saving and avoided the formation of toxic by-products. In particular, high dosages of PAA lead to the release of H_2O_2 and acetic acid, which cause an increase in BOD and COD concentration in the effluent, furthermore percentages of still active reagent would be released in the water body receptor. A recent study demonstrates that values of residual disinfectant higher than 2 mg/L of PAA, cause acute toxicity on *Daphnia magna* and *Vibrio fischeri* (Collivignarelli 2017).
Table 2.6.9. reports the concentration of the investigated microbiological parameters after physical and chemical disinfection treatments and their removal in terms of logarithmic units. According to the almost complete removal of the protozoa and the complete removal of Salmonella obtained during the biological treatment, their presence was not detected after the physical and chemical disinfection processes.

		UV 40 mJ/cm^2	Peracetic Acid 1 mg/L
Total Coliforms	[MPN/100 mL]	5.6 ± 6.2 × 10	2.2 ± 4.2 × 10
	LUR	2.0 ± 0.5	2.5 ± 0.9
E. coli	[MPN/100 mL]	5.6 ± 5.3	<0.5 *
	LUR	1.8 ± 0.2	2.6 ± 0.5
Somatic coliphages	[PFU/100 mL]	5.7 ± 5.7	2.1 ± 1.4 × 10
	LUR	2.1 ± 1.1	1.5 ± 0.3
Clostridium perfringens	[CFU/100 mL]	2.4 ± 3.1 × 10^3	2.2 ± 3.1 × 10^3
	LUR	0.05 ± 0.19	0.06 ± 0.11

***** Under detection limit, *E. coli*. never detected in 200 mL.

Tab. 2.6.8. Performances of the physical and chemical disinfection processes in terms of concentrations of the investigated microbiological parameters and removal efficiencies.

The results obtained for total coliforms and *E. coli* indicate that both investigated disinfection processes were very efficient in removing these groups of bacteria. After disinfection with UV rays was obtained an effluent with an average value of 56 MPN/100 mL for total Coliforms and 5.6 MPN/100 mL for *E. coli.* Chemical disinfection with 1 mg/L of peracetic acid showed similar efficiency as UV with regard to total coliforms and resulted even more effective against *E.coli*. In particular, was obtained an effluent with a mean value of 22 MPN/100 mL for total Coliforms and less than 0.5 MPN/100 mL for *E. coli.* Indeed, the latter was never detected in 200 mL.

In both disinfection processes the concentrations of *E. coli* meets the Italian limit for agricultural wastewater reuse (i.e., ≤10 CFU/100 mL). Normally, for conventional treatment, the dosages of peracetic acid and UV are much higher. For example Gori and Caretti (2008) to meet the Italian limit of *E. coli* in a study on two traditional municipal WWTPs (composed of preliminary, primary, secondary and sand filtration treatments) used 2–4 mg/L of PAA followed by UV at the dose of 165–170 mJ/cm^2. In another study Caretti and Lubello (2003) show that it is necessary a disinfection with 2 ppm of PAA followed by 192 mJ/cm^2 UV to have a removal of 6 logs of *E.coli.*

The above results indicated that PAA is more suitable than UV for *E.coli* removal and has the same efficiency on total coliforms.
On the contrary Somatic Coliphages were more sensitive to UV than PAA, in fact the concentrations of these viruses in the effluent was on average 5.7 PFU/100 mL after physical disinfection and 2.1 × 10 after the chemical one. The greater somatic coliphages disinfection capability of UV radiation than PAA is also reported by Gehr et al. (2003) The authors found that the removal of 1 log unit of MS2-coliphages could be achieved with a UV dose of 20 mJ/cm^2, and the removal of this virus linearly increased with the increase of the UV dose. Differently, the same virus removal of about 1 log unit was obtained by using PAA doses in the range 1.5–4.5 mg/L.
As regards the *Clostridium perfringens* spores, the results obtained during the disinfection tests showed their greater resistance compared to the other indicators. In fact, the treatment with both UV and PAA did not produce a significant reduction in the number of spores compared to the effluent of the SBBGR system. Taking into account the low removal of *C. perfringens* obtained, additional tests at higher doses of PAA (i.e., 5, 10 and 20 mg/L) were carried out. The results indicated that a PAA dose of 20 mg/L allowed the complete removal of *C. perfringens*. However, such a high dose is not economically sustainable; moreover it would lead to a residual COD concentration in the effluent, which is not compatible with wastewater reuse.

2.6.4. Treatment performance evaluation in terms of ARGs

Beside the above described indicators of faecal contamination, in order to fully evaluate the water quality for reuse, antibiotic reresistance genes have been investigated.
The presence and removal of ARGs, and total bacteria (16S rDNA) were analysed. In particular,were collect samples of raw wastewater, SBBGR effluent and sand filtration effluent. Finally, for the evaluation of disinfection treatment effect on ARGs, disinfection tests were performed after the applied UV and PAA disinfection.
The most widely used molecules in antibiotic prophylaxis are divided into different classes, including: Betalactam, Macrolide, Sulfonamide, Tetracycline, Vancomycin. Nine selected genes (*ampC, mecA, ermB, sul1, sul2, tetA, tetO, tetW, vanA*) encoding for the resistance to the five classes of antibiotics, were analysed in the SBBGR influent. Most of

the selected genes were indexed in other studies as reference genes for monitoring antibiotic resistance in the environment (Berendonk et al., 2015). Only four ARGs, namely *ermB, sul1, sul2 and tetA*, were detected during sampling period (280 days). The ARGs detected in our samples were previously reported in wastewater or WWTPs effluents (Chen and Zhang, 2013; Du et al., 2014;). In particular, these studies report the widespread presence in the raw wastewater of the *sul* genes that show resistance to the sulphonamide antibiotic class. The very rapid dissemination of resistance to these antimicrobial agents is the reason for which the use of sulfonamides has been limited since the mid-1990s (Sköld, 2000). The easy of diffusion is probably due to the fact that *sul1* and *sul2* are present on very efficient vehicles for dissemination. Among the three tested tetracycline resistance genes, only *tetA* was detected in the analysed samples. *ErmB* gene that confers cross-resistance to macrolide was detected also.

	Wastewater	**SBBGR effluent**	**SF effluent**
16S rDNA (GC/mL)	$1.59\times10^{9} \pm 2.2\times10^{7}$	$3.45\times10^{7} \pm 5.1\times10^{5}$	$8.9\times10^{6} \pm 9\times10^{5}$
LUR		1.5 ± 0.5	0.9 ± 0.5

Tab. 2.6.9. Abundance and logarithmic removal of total bacteria (16S rDNA) in wastewater, SBBGR effluent and after sand filtration.

In Table 2.6.9. the level of 16S rDNA before and after the biological treatment and sand filtration is shown together with the average logarithmic removal. SBBGR treatment allows a decrease of 1.5 logs of 16S rDNA GC/mL, this removal cability is also obtained in other biological plants, in fact Wery et al. (2008) using qPCR to quantify total bacteria (16S rDNA), in a French WWTP, has verified a reduction of 2 logs after secondary treatment. The results obtained confirm the good performance of the SBBGR for the reduction of microbial contamination reported in the previous paragraphs. An enhancement of total bacteria reduction was observed after sand filtration, with removal values of 0.9±0.5 log units for 16S rDNA. The lower removals observed in our investigation, might be due to the use of qPCR method that, differently from the cultivation- based methods, detects also dead bacteria and thus cannot shows bacterial inactivation processes.

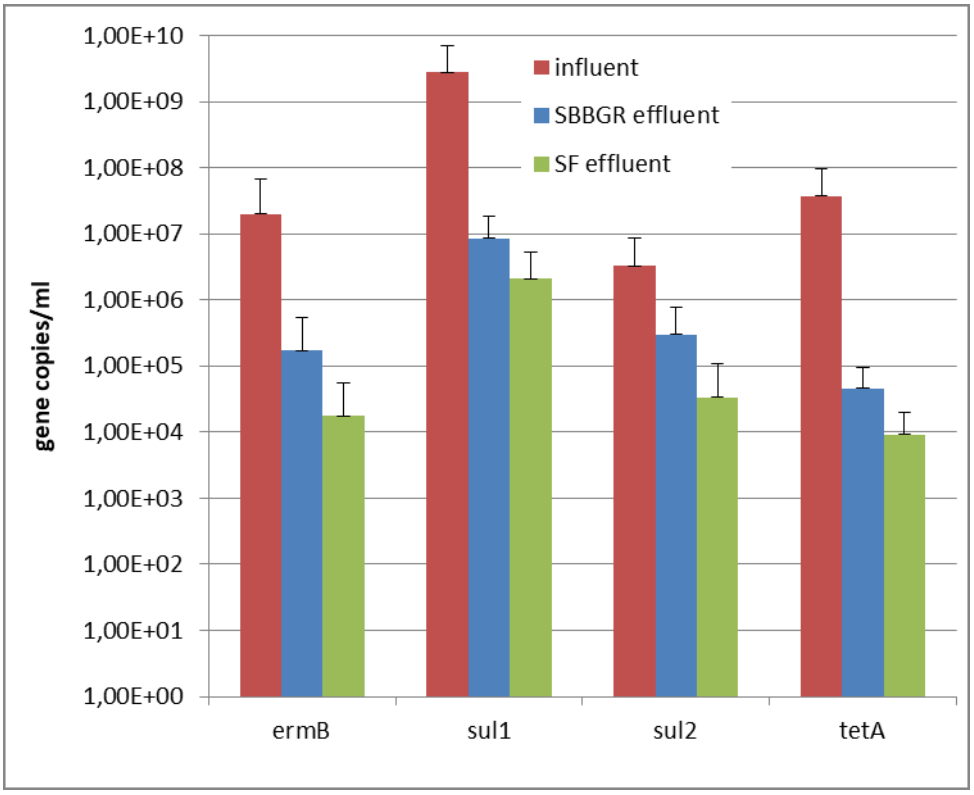

Fig. 2.6.13. Levels of *ermB*, *sul1*, *sul2*, *tetA* in influent, SBBGR effluent and after sand filtration.

A evaluation of the concentration of the four genes detected was made in influent, SBBGR effluent and after sand filtration (fig 2.6.13.). The ARGs were present in high concentrations in the raw waste but also in the effluent after the treatment line. These results confirm that wastewater and treatment systems can be a source of antibiotic resistance in the environment due to the favorable conditions present, such as the presence of nutrients and the close contact between the microbial population.

Mean concentrations of ARGs in the raw waste ranged from a minimum value of 3.2 ± 5.3 $x10^6$ recorded for *sul2* and a maximum of 2.7 ± 4.3 $x10^9$ of *sul1* and are reduced in a range from 4.6 ± 4.7 $x10^4$ of *ermB* to 8.5 ± 10 $x10^6$ of *sul1.* The SBBGR system is able to reduce 1-2 logarithmic units of ARGs. In particular, a removal of 1.6 ± 0.7 LUR was recorded for *ermB*, 1.9 ± 0.8 LUR for *sul1* and 2.2 ± 1.1 for *tetA*, while for *sul2* there was a

lower decrease compared to the other genes (1.0 ± 0.4 LUR), underlining the difference in behavior of this gene compared to the others.

Sand filtration was applied for the enhancement of SBBGR effluent quality, in fact an improvement of ARGs reduction was observed. In particular, removal values of 1.1 ± 0.7 log units for *ermB*, 1.1 ± 0.8 log units for *sul1*, 1.3 ± 0.8 log units for *sul2 and* 0.9 ± 0.7 log units for *tetA* have been registered. In agreement with these results, reduction efficiencies of approximately 1 log unit were reported by other authors that used qPCR to investigate ARGs removal by sand filtration in drinking water treatment plants (DWTPs) (Xu et al., 2016). The good removal efficiency obtained after sand filtration is in agreement with the excellent performance obtained by this technology for the removal of suspended solids, fecal indicators and some pathogens, described above. Overall, removal level of 2-3 log units for ARGs were achieved in the SBBGR combined with sand filtration.

Limited or no decrease of ARGs normalized to 16S rDNA is shown in figure 2.6.14., this result suggests that the removal of ARGs in the SBBGR system is mainly due to the removal of total bacteria. Auerbach et al. (2007) report silmilar results for a study concerning the reduction of ARGs in activated sludge systems, showing that there is no significant difference in the ratio of ARGs concentration compared to total bacteria DNA, in wastewater and after biological treatment. This result shows that the treatment system does not seem to promote an enrichment of ARGs among bacteria during the whole process.

ErmB relative concentration in the effluent is of the same order of magnitude of the influent one, in fact an average value of 5.5 $x10^{-3}$ ± $1x10^{-2}$ was recorded in the raw wastewater and 3.8 ± 8 $x10^{-3}$ in the treated one. The same occurred for *sul1* with average concentrations of 4.3 $x10^{-1}$ ± 1.2 x10 in the influent and 3 ± 2.6 $x10^{-1}$ in the treated water. A greater decrease of ARGs relative concentration was observed only for *tetA,* reporting 2 ± 2.7 $x10^{-2}$ averege value in wastewater and 1.9 ± 1.4 $x10^{-3}$ after the treatment. Moreover the relative abundance of *sul2* increased after the treatment, for this gene the relative average concentrations change from 1.1 ± 2 $x10^{-2}$ in the influent to 2.2 ± 3.9 x 10^{-2} in the effluent. As shown for the removal values of the ARGs, also in this case *sul2* shows a different behavior with respect to the other genes. The relative abundance values of *sul2* after SBBGR treatment suggest that this gene has a greater ability to transfer.

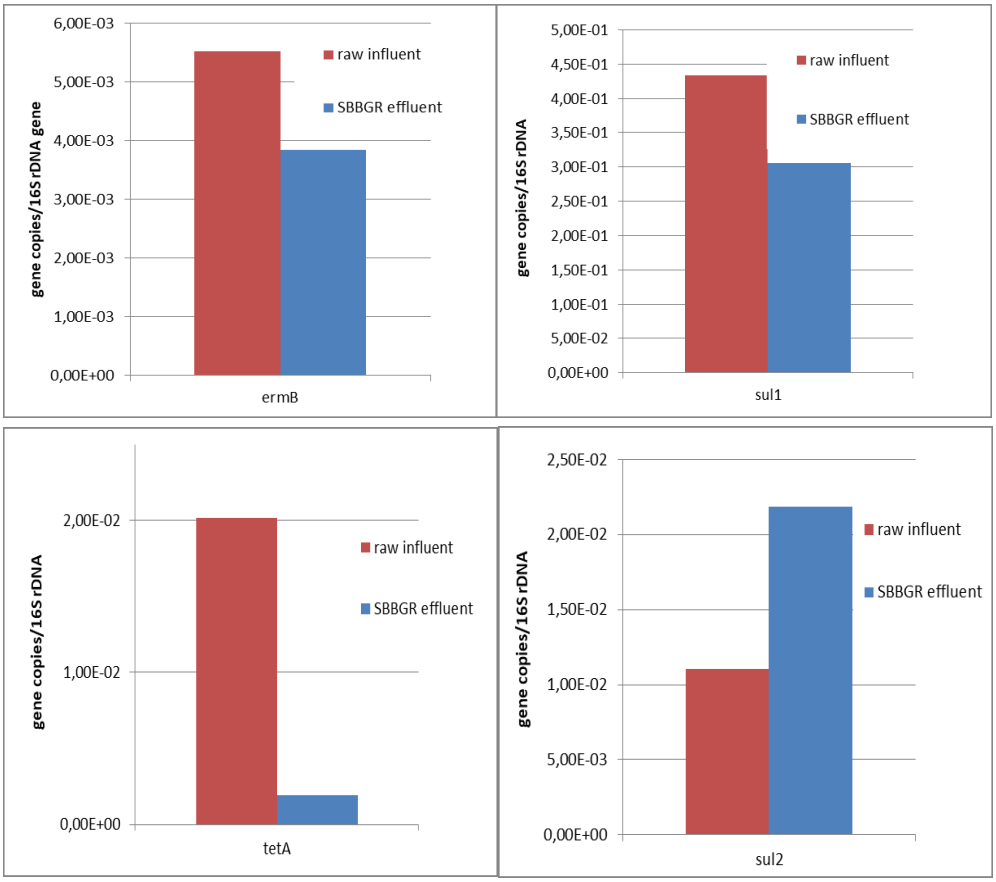

Fig. 2.6.14 ARGs(*ermB*, *sul1*, *sul2*, *tetA*) levels normalized on 16S rDNA genes abundance in influent and SBBGR effluent.

To better assess the fate of ARGs in the treatment plant, the level of the four studied genes was also analyzed in the SBBGR biomass evaluating the number of gene copies per gram of dry weight. In table 2.6.10. it is possible to compare the abundance of ARGs in the biomass and in the raw wastewater. *ErmB, sul1, sul2 and tetA* were all detected in the biomass in amount ranging from $4.7 \pm 4.1 \times 10^6$ to $3.3 \pm 2.3 \times 10^8$. The ARGs content in the biomass results for *ermB, sul1, sul2 and tetA* less than that measured in the influent, this result suggests that in the biological system (i.e. within the biofilter) there is no selective promotion of ARGs and there is not even enrichment of the bacterial population harbouring ARGs.

	ermB	*sul1*	*sul2*	*tetA*
Influent	6.7 ± 16x10^{11}	9.2 ± 15 x10^{12}	1.1 ±1.8x10^{10}	1.2 ± 2.0 x10^{11}
Biomass	8.7 ± 10 x 10^{6}	3.3 ± 2.3 x10^{8}	4.7 ± 3.1 x10^{7}	4.7 ± 4.1 x10^{6}

Tab 2.6.10 ARGs concentration in influent and SBBGR biomass are given in CG/g dry weight.

On three different effluent samples after sand filtration, disinfection tests were carried out with UV (40 mJ/cm^2) and peracetic acid (1 mg/L). Although these methods were found to be effective for the reduction of some faecal contamination indicator organisms, no relevant effect was found on ARGs (figure 2.6.15). To our knowledge In literature, few studies on the effect of PAA disinfection on ARGs are reported. In a study on combined sewer overflows, Eramo et al. (2017) shows that a concentration of 5 mg/L of PAA for 20 min is needed to obtain 1 logarithmic unit removed. Considering the diversity of the treated waste the PAA concentrations used are however greater than those tested in this thesis work. Other studies have evaluated the efficiency of ARGs removal after UV treatment, reporting limited or no effect. According to the results obtained, Auerbach et al., 2007 shows that the number of detected genes resistant to tetracycline has not decreased with 30-100 mWs/cm^2 UV dosage. Munir et al. (2011) reported that UV disinfection had no effect on ARGs levels in five different WWTPs. McKinney and Pruden (2012) suggest that the detection by qPCR of the damage caused by UV rays on DNA could be difficult when, as in this experimentation, short amplicons are used. However, the same authors show that if long-amplicons are used, a high dose of UV (200-400 mJ/cm^2) is still necessary to reduce the amount of ARGs in the effluent.
Overall, ARGs were still present in the final treated effluent for agricultural reuse ranging from10^3 to 10^6 GC/mL.

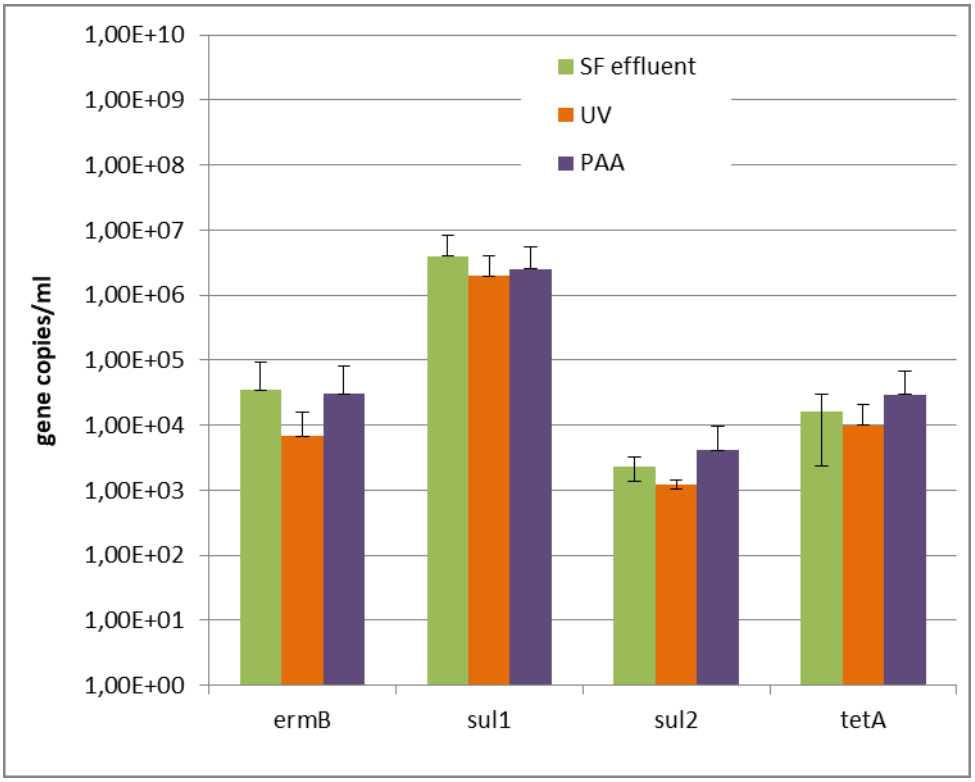

Fig. 2.6.15. Levels of *ermB*, *sul1*, *sul2*, *tetA* in effluent after sand filtration, UV and PAA disinfection treatment.

2.6.5 General economic evaluation

The need to comply with regulatory limits must be combined with the adoption of adequate process systems, allowing cost limitation. In case of reuse of treated wastewater, is necessary an economic assessment that takes into account both the costs of planting and operation, but above all the economic benefit in terms of saving water resources and "non-discharge" waste.

The specific electric energy consumption of the biological SBBGR treatment was about 0.39 kWh/m^3 of treated wastewater. This value is in line with those reported in the literature for conventional wastewater treatment plants based on activated sludge process (0.45 kWh/m^3) but lower compared to oxidation ditch systems (0.77 kWh/m^3) (Lazarova et al., 2012). Assuming an electricity cost of 18 euro cents per kWh (electricity costs for Ital-

ian industrial consumers obtained from Eurosat 2018), a cost of 7.02 euro cents per m^3 of wastewater results. The cost of physical or chemical disinfection, however, should be added to this value. Taking into account the operative conditions (i.e., contact time of 1 s) and features (i.e., working volume of 0.5 L and power supply of 40W) of UV lamp used, a specific energy consumption of only 0.02 kWh (or 0.36 euro cents) per m^3 of wastewater disinfected results. Energy consumption of physical disinfection by UV lamp is lower than the cost of peracetic acid in the chemical disinfection. In fact, on the basis of PAA dosage (i.e., 1 g per m^3 of wastewater) and price (i.e., 20–40 euro/kg), a cost of 2–4 euro cents per m^3 of wastewater disinfected is obtained. These data clearly show that in the case of UV disinfection the relative cost can be considered negligible compared to that of biological treatment by SBBGR.

For SBBGR system more of the electric energy demand was used for the oxygen supply and for recirculation pump but SBBGR was highly effective in reducing the amount of waste sludge, allowing considerable savings about costs concerning sludge management. Literature data (Andreoli et al., 2007) show that sludge treatment and disposal costs can account for up to 60% of total operating costs in a traditional WWTP, for this reason the reduction of sludge production it is a considerable economic advantage for SBBGR system.

3.0 CONCLUSIONS

The present thesis work highlights the validity of the innovative biological degradation process SBBGR for the treatment and reuse of raw urban wastewater.

In this study it is shown that SBBGR system and its integration with different disinfection strategies (sand filtration, UV irradiation, PAA addition) are effective to produce an effluent that conforms to the stringent standards required in Italy for agricultural reuse.

The biological treatment based on SBBGR is able to ensure a stable and effective removal of suspended solids and organic matter with average removal of 98% of TSS, 92% of COD and 99% of BOD, respecting the limit set by national legislation for crops irrigation.

The SBBGR system is also able to ensure excellent nitrogen removal efficiency through the biological nitrification and denitrification process. In particular, the ammonia removal is on average 99% and that of TKN of 95%, with residual concentrations always lower than 4 mg/L, highlighting the presence of a very stable nitrification process. The total nitrogen data (average removal of 78%) shows also the presence of a denitrification process.

SAR, pH and conductivity values recorded for the SBBGR effluent, show that the water treated by the system is suitable for irrigation, ensuring the preservation of the soil without resorting to additional technologies.

Moreover the plant was characterised by a very low sludge production, during the study period a sludge production value of approximately 0.13 kg of SST per kg of COD removed was calculated. This value is much lower than that of conventional treatment systems (about 80% lower) and represents a further advantage of the SBBGR system.

The disinfection performances of the SBBGR were higher than that of conventional municipal wastewater treatment plants in particular for the *E. coli* content. The concentration found in the effluent of the SBBGR system is lower than the limit set by Italian legislation for the discharge into surface waters (i.e. 5000 UFC/100 mL) but higher than the limit for agricultural wastewater reuse. Respecting the Italian regulations for irrigation water, Salmonella has not been detected in any effluent sample treated by the SBBGR system.

The high quality of the effluent both for the gross parameters and the microbiological point of view, allows to reach the stringent standards foreseen for the reuse, using a simple multi barrier treatment scheme with very low dosage of disinfecting agents.

The public opinion is one of the main limitations to the reuse of wastewater, the greatest concern is the exposure to pathogens. The safety of wastewater treated with the SBBGR system is demonstrated by investigating microbiological indicators in addition to those normed.

The SBBGR plant followed by sand filtration completely removes *Cryptosporidium* and *Giardia* which are generally highly resistant.

SBBGR showed removal efficiencies of *C. perfringens* and somatic coliphages comparable to those of conventional WWTPs, but the efficiency of removal improves simply after sand filtration whit an overall removal of 2.7 and 3.2 log units respectively.

The disinfection tests performed on the effluent of the SBBGR system show that UV radiation and PAA doses as low as 40 mJ/cm^2 and 1 mg/L respectively were able to reduce *E. coli* content in the final effluent below the limit for agricultural reuse in Italy (i.e., 10 CFU/100 mL).

Somatic Coliphages are more sensitive to UV than PAA, whereas both physical and chemical treatment does not produce a significant reduction in the number of spores compared to the effluent of the SBBGR system.

With regard to the antibiotic resistance genes, a high concentration is found in the raw waste water and despite the ability to remove of SBBGR system these genes are still present also in the effluent. The reduction of ARGs improves by combining the SBBGR system with sand filtration showing removal level of 2-3 log units, while limited effect of UV and PAA on ARGs are shown.

At present, concentrations that represents a risk for human health are not known, so it is necessary to deepen research on these genes, their behavior in the environment and the impact of wastewater reuse.

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6.0 ANNEX

Annex 1. Quality limits for wastewater reuse in Italy (D.M. 185 – 2003).

	Parameter		**Limit**
Physical and chemical parameters (The reported limits refer to the mean values during one year operation. Each individual sample value cannot exceed 100% of the limit)	pH		6-9.5
	SAR		10
	Coarse materials		absent
	TSS	mg/L	10
	BOD_5	mgO_2/L	20
	COD	mgO_2/L	100
	Total phosphorus	mgP/L	2-10*
	Total nitrogen	mgN/L	15-35*
	Ammonia	$mgNH_4/L$	2
	Conductivity	µS/cm	3000
	Aluminium	mg/L	1
	Arsenic	mg/L	0.02
	Barium	mg/L	10
	Beryllium	mg/L	0.1
	Boron	mg/L	1.0
	Cadmium	mg/L	0.005
	Cobalt	mg/L	0.05
	Total chromium	mg/L	0.1
	Chromium VI	mg/L	0.005
	Iron	mg/L	2
	Manganese	mg/L	0.2
	Mercury	mg/L	0.001
	Nickel	mg/L	0.2
	Lead	mg/L	0.1
	Copper	mg/L	1
	Selenium	mg/L	0.01
	Tin	mg/L	3

	Thallium	mg/L	0.001
	Vanadium	mg/L	0.1
	Zinc	mg/L	0.5
	Total Cyanides (as CN)	mg/L	0.05
	Sulphides	mg/L	0.5
	Sulphites	mg/L	0.5
	Sulphates	mg/L	500
	Active chlorine	mg/L	0.2
	Chorides	mg/L	250
	Fluorides	mg/L	1.5
	Animal/Vegetable fats and oil	mg/L	10
	Mineral oils	mg/L	0.05
	Total phenols	mg/L	0.1
	Pentachlorophenol	mg/L	0.003
	Total aldehydes	mg/L	0.5
	Tetrachlorethylene + trichlorethylene	mg/L	0.01
	Total chlorinated solvents	mg/L	0.04
	Trihalomethanes	mg/L	0.03
	Total aromatic organic solvents	mg/L	0.01
	Benzene	mg/L	0.001
	Benzopyrene	mg/L	0.00001
	Total nitrogenous organ-ic solvents	mg/L	0.01
	Total surfsctants	mg/L	0.5
	Chlorinated pesticides	mg/L	0.0001**
	Phosphorus pesticides	mg/L	0.0001**
	Other pesticides	mg/L	0.05

Microbiological parameters	*E.coli*	CFU/100mL	10***
	Salmonella		Absent

* In case of agricultural reuse and on the base of regional regulations.

** For each specific pesticide.

*** In 80% of the samples without exceeding 100 CFU/100 mL

7.0 CURRICULUM

Dr. Silvia Chimienti received the Master degree in Industrial and Environmental Biotechnology at the University of Bari "Aldo Moro", discussing an experimental thesis entitled "High efficiency and low sludge production system for urban wastewater treatment". Since 2015, she is a PhD student in "Environmental, Territorial and Construction Risk and Development" at the Polytechnic of Bari in the "Science and Materials Technology" field. Furthermore, since 2014 she is a Researcher Fellow of the Water Research Institute of Italian National Research Council, Department of Bari.

Silvia has experience in wastewater treatment and, in particular, in aerobic granular biomass technologies. During the research period, Silvia has gained experience in the management of laboratory scale plants and pilot scale plants, developing skills in monitoring physical,chemical and microbiological parameters of wastewater. Furthermore, she has acquired expertise in the treatment of urban wastewater both with biological systems and with chemical and physical enhancement.

Thanks to the participation in several research projects, she has been able to explore issues such as novel processes for wastewater treatment, integration of chemical and biological oxidation processes for industrial wastewater, anaerobic processes, ozone processes, biomass characterization.

The following scientific publications were produced as part of the research activities:

1. De Sanctis M., Del Moro G., Bellifemine D., Chimienti S., Ritelli P., Di Iaconi C. “Valorizzazione energetica dei residui di posidonia spiaggiata mediante digestione anaerobica potenziata chimicamente”. Capitolo 4, pp. 275-279 in “La ricerca sulle acque e le nuove prospettive do valorizzazione dei risultati in ambito pubbli-

co e privato", Brugnoli E., Uricchio V.F. (2016) Cacucci Editore, Bari. IBSN: 9788866115168.

2. De Sanctis M., Del Moro G., Bellifemine D., Chimienti S., Ritelli P., Di Iaconi C. "Un sistema avanzato per il trattamento e il riuso delle acque reflue in agricoltura". Capitolo 4, pp. 309-314, in "La ricerca sulle acque e le nuove prospettive do valorizzazione dei risultati in ambito pubblico e privato", Brugnoli E., Uricchio V.F. (2016) Cacucci Editore, Bari. IBSN: 9788866115168.
3. De Sanctis M., Del Moro G., Chimienti S., Ritelli P., Levantesi C., Di Iaconi C. (2017) Removal of pollutants and pathogens by a simplified treatment scheme for municipal wastewater reuse in agriculture. Science of the Total Environment 580:17-25.
4. Piergrossi V., De Sanctis M., Chimienti S., Di Iaconi C. (2018) Energy recovery capacity evaluation within innovative biological wastewater treatment process. Energy Conversion and Management 172, 529-539.
5. Muñoz I., Rosiek S., Portillo F., Batlles F. J., Martínez Del Río J., Acasuso I., Piergrossi V., De Sanctis M., Chimienti S., Di Iaconi C. Prospective environmental and economic assessment of solar assisted thermal energy recovery from wastewater through a sequencing batch biofilter granular reactor. Journal of Cleaner Production (in press).