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Petrochemical slop wastewater treatment by means of aerobic granular sludge: effect of granulation process on bio-adsorption and hydrocarbons removal

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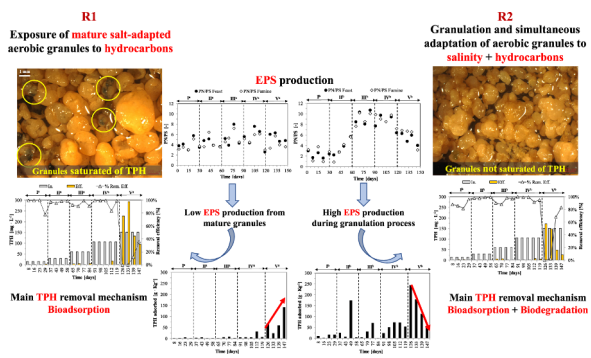
HIGHLIGHTS

- Aerobic granular sludge technology is suitable for recalcitrant wastewater treatment.
- Mature granules subjected to slop do not easily adapt to the recalcitrant wastewater.
- Bio-adsorption is the main removal pathway when using mature granules to treat slop.
- EPS production during granulation enhanced the particulate bio-adsorption.
- Granulation with slop favours biological adaptation of bacteria to hydrocarbons.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this work is to study the mechanism for hydrocarbons removal from slop wastewater by means of aerobic granular sludge technology (AGS). Two sequencing batch reactors (SBRs), R1 and R2, worked for 150 days according to two different strategies: (i) slop treatment with mature salt-adapted granules (R1); (ii) cultivation of AGS with slop wastewater for its treatment (R2). Results revealed that, despite the similar physical properties of AGS at the end of experimental period (4.4–4.8 $gTSS \cdot L^{-1}$ and 2.2–2.5 $gVSS \cdot L^{-1}$, and mean dimensions of 1.15 mm and 1 mm, for R1 and R2 respectively), in R2 granules highlighted better total petroleum hydrocarbons (TPHs) removal efficiencies than R1 (83% vs 36%), when real slop was fed to the reactors. The direct cultivation of AGS with slop (R2) prompted a higher extracellular polymeric substances (EPSs) production that enhanced the bio-adsorption of TPH, thus favouring the adaptation of bacteria to hydrocarbons.

1. Introduction

Nowadays sea pollution due to the direct discharge of shipboard wastewater is an issue of relevant environmental impact. Slop wastewaters are produced from the activity of washing of oil tankers with seawater and their direct discharge to the sea is strictly prohibited by IMO-Marpol 73/78 regulation [1]. This kind of industrial wastewater is mainly characterized by high salinity and high recalcitrant fraction composed by hydrocarbons. Slops treatment is not easy to achieve mainly due to their heterogeneous composition, and the presence of

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emulsified oils. Chemical processes are commonly used to treat industrial wastewater characterized by recalcitrant compounds, ensuring high removal efficiencies but they imply high operational costs, related to the use of chemicals, and a considerable environmental impact [2,3]. On the other hand, biological processes are environmental-friendly but, at the same time, they could not be effective in presence of recalcitrant and toxic pollutants as well as at high salinity operational conditions due to inhibition effects towards microbial cells [4–7]. Aerobic granular sludge (AGS) technology is a particular biofilm system where microorganisms are self-aggregated in granules [8] and it appears as one of the most promising biotechnologies able to treat industrial recalcitrant wastewater [2,9] both as synthetic [10–13], and real wastewater from various sectors (slaughter house, rubber industry, palm oil mill effluents, brewery, winery, dairy industry, landfill leachate) [14–20]. Recently, an emerging research topic relating the AGS technology is the peculiarity of granules to bio-adsorb the particulate fractions of industrial wastewater, thus increasing the contact time between microorganisms and substrates. This could promote the hydrolysis and biodegradation of the more recalcitrant fractions [2]. The bio-adsorption of some pollutants on cell surfaces of microorganisms forming aerobic granules, is mediated by the extracellular polymeric substances (EPSs) that help the binding of recalcitrant compounds with the biopolymers produced by microbial cells [21–27]. The treatment of petrochemical wastewater with AGS aroused interest of some researchers. Carucci et al. (2010) [12] treated wastewater contaminated by 4-chlorophenol by using acetate as co-substrate to favour co-metabolism. Although this was an attractive way to treat recalcitrant substrates, its application could be too expensive at full-scale treatment plants, due to the costs of the reagents for co-metabolic substrate. A similar approach was applied by Zhang et al. (2011) [13] that treated petrochemical wastewater from an oil refinery and concluded that when only petrochemical wastewater was fed to the reactor, both granules properties and performances deteriorated. Therefore, they suggested to add co-metabolic substrate, such as sodium propionate, as a technique to ensure effective removal of nutrients and maintain compact structure of aerobic granules.

In a previous experience, the authors studied the possibility to cultivate granular sludge with petrochemical wastewater characterized by high salinity [28]. The aerobic granules were fed with a mixture of real slop wastewater, and simulated slop wastewater made as a mixture of diesel and sodium acetate, resulting in an overall low total petroleum hydrocarbon (TPH) concentration next to 6.8 $mgTPH \cdot L^{-1}$. They found that a rapid granulation occurred due to a high EPS production that enhanced the aggregation of microorganisms. Moreover, in a recent research, the same authors proposed a model of granules bio-adsorption for hydrocarbons [10] of low strength TPH load wastewater (6.5 $mgTPH \cdot L^{-1}$) obtaining TPH removal in compliance with limits (TPH concentration in the effluent was lower than 5 $mg \cdot L^{-1}$). However, by mixing slop wastewater with a readily biodegradable fraction such as sodium acetate, it is not possible to exclude a co-metabolism effect in the hydrocarbon removal mechanism. Caluwé et al. (2017) [29] proved that it is possible to obtain aerobic granules from flocculent sludge by treating a petrochemical wastewater without any external carbon source. However, since with the word petrochemical it is possible including a high variability of compounds, Caluwé et al. (2017) [29] treated a wastewater rich in monoethyleneglycol (MEG), methanol (MeOH) and ethanol (EtOH) that are bioderadable organic compounds. In a more recent study, it was demonstrated that the AGS could form treating synthetic petroleum wastewater, performing good COD and oil removal efficiencies [30]. However, studies on a synthetic wastewater do not offer a global view towards the effects of a real industrial wastewater. Real petrochemical slop wastewater such that treated in the present study, had a complex composition and they were mainly composed by recalcitrant hydrocarbons with high molecular weight, falling into the diesel range organic (DRO). The treatment of this kind of wastewater is the real novelty of this work. The main objective of this study is to compare two strategies for the treatment of high-strength TPH and saline slop wastewater: (i) using mature aerobic granules previously adapted to salinity, (ii) cultivating aerobic granules directly with slop. In the first case, mature salt-adapted granules were acclimatized to hydrocarbons while, in the second case, granules were simultaneously adapted to a gradual increase of salinity and hydrocarbons. Attention was paid to TPH removal, bio-adsorption mechanism and EPSs production.

2. Materials and methods

For all the analytical procedures, quality assurance/quality control (QA/QC) in this study was guaranteed through: – the adoption of standard methods for each tested parameter; – a constant training of all workers in the laboratory according to the standard methods; - logging and reviewing of all sampling and analytical steps to be sure no error occurs. Regarding the sampling procedures, all fresh samples were collected and analysed in triplicates. For each chemical analysis, blanks samples and reference samples were analysed to validate the measurement.

2.1. Bench scale plant description and operational conditions

Two sequencing batch airlift reactors (SBARs), hereinafter called R1 and R2, with a working volume of 3.5 L and a H/D ratio of 10, worked for 150 days of operation at the Laboratory of Sanitary and Environmental Engineering of University Kore of Enna (Italy). The reactors were the same used by Campo et al. (2018) [4] (Fig. S1). In steady state conditions, the reactors were operated in 6-hours cycles comprising: 20 min feeding, 320–333 min aeration, 2 min settling and 5 min effluent discharge. The volumetric exchange ratio (VER) was fixed at 50% and the hydraulic retention time (HRT) was kept at about 12 h.

2.2. Seed sludge and operational strategy

The two SBARs were seeded with different biomasses. R1 was seeded with 1.75 L of salt-adapted mature granules grown according to a step-wise increase of salinity, whose characteristics were described in a previous experimental application [4]. R2 was seeded with 1.75 L of flocculent activated sludge collected from the municipal wastewater treatment plant (WWTP) of Enna (Sicily, Italy), operating with a conventional activated sludge (CAS) system. Therefore, the operational strategy consisted of treating slop wastewater with different seed sludge [31]. In R1, granules previously cultivated and adapted to salinity had undergone the gradual increase of hydrocarbons concentration of slop. In R2 granules were cultivated with petrochemical slop wastewater starting from flocculent sludge, therefore subjected to a simultaneous increase of salinity and hydrocarbons. Before the slop dosage, the flocculent seeding biomass of R2 was subjected to a short preliminary adaptation phase (15 days) to avoid organic and hydraulic loading shocks (data not shown). At this purpose, the organic loading rate (OLR) was gradually increased from 0.4 to 1.6 kg $COD \cdot m^{-3} \cdot d^{-1}$, and the settling time was gradually decreased from 15 min to 2 min.

2.3. Wastewater composition and operational conditions

Petrochemical shipboard slops were collected from barges located in an oil costal deposit in the Augusta harbour in Sicily (Italy). As many industrial wastewaters, slops were characterized by a high variability depending on the kind of product transported by the oil tanker (gasoline, diesel, gas, crude oil, etc.…). Before to characterize and treat the slop, a preliminary treatment of de-oiling and gravity separation was performed, as suggested in the BREF (Bat Reference Document) for "Refining of Mineral Oil and Gas" [32], in order to remove a large portion of floating oil and greases. Slops, after de-oiling pre-treatment, were mainly characterized by a total chemical oxygen demand (COD_T)

Table 1
Average composition of slop wastewater.

Parameter	Unit	Value
COD_T	mg L^{-1}	816 ± 94
COD_S	mg L^{-1}	530 ± 32
TOC	mg L^{-1}	390 ± 3
TPH	mg L^{-1}	151 ± 3
TSS	mg L^{-1}	352 ± 84
NaCl	g L^{-1}	37.8 ± 1.5
Br^-	mg L^{-1}	412 ± 13
SO_4^-	g L^{-1}	3.5 ± 0.1
pH	–	7.78 ± 0.62

of 816 ± 94 mg·L^{-1}, a soluble chemical oxygen demand (COD_S) of 530 ± 32 mg·L^{-1}, a total organic carbon (TOC) of 390 ± 3 mg·L^{-1}, total petroleum hydrocarbons (TPHs) of 151 ± 3 mg·L^{-1}, salinity of 37.8 ± 1.5 g $NaCl^-$·L^{-1} and a pH of 7.78 ± 0.62. Table 1 resumes the main slop characteristics after de-oiling and gravity separation. A typical chromatogram of de-oiled slop and the hydrocarbon speciation are shown in Fig. S2 and it is possible to see that the hydrocarbons range is mainly represented by Diesel Range Organic (DRO). This is in keeping with the crude oils hydrocarbons speciation.

The experimental period was composed of five phases of 30 days-duration each, resumed in Table 2, where the average OLR was subjected to small experimental variations due to the real flows rates influent to the reactors.

In R1, the influent was characterized by a step-wise increase of slop dosage and, since the inoculum was constituted by salt-adapted granules, the salinity was maintained constant to the value of real slop by dosing sodium chloride until the Phase IV^a. In R2, the feeding was also characterized by step-wise increase of slop dosage but, since the inoculum was flocculent sludge from a CAS system, in this case the reactor was subjected to a simultaneous increase of both salinity and hydrocarbons in order to adapt the biomass to the saline environment and to the recalcitrant substrate. Therefore, to promote a rapid and stable granulation [33], an almost constant OLR next to 1.6–1.7 KgCOD·m^{-3}·d^{-1} was maintained throughout all the experimental period. A mixture of synthetic wastewater and the real slop wastewater was dosed, with a gradual decreasing volume of the synthetic medium in each Phase until the Phases IV^a and IV^b. The synthetic wastewater was characterized by the following stoichiometric proportions [34]: $C_2H_3NaO_2$ 97.7 mM, $MgSO_4·7H_2O$ 3.7 mM, K_2HPO_4 20 mM, KH_2PO_4 10 mM, KCl 4.8 mM, NH_4Cl 30 mM. This medium was diluted with tap water to obtain the desired OLR for the whole experimental period.

In Phases V^a and V^b no more synthetic medium was dosed, and the sole carbon source was provided by a 100% of volume of real slop wastewater fed to both the reactors during the last 30 days of operation. Moreover, since slop wastewater is lacking in nutrients that were necessary for metabolism of bacteria, nitrogen and phosphorus were added as NH_4Cl, K_2HPO_4 and KH_2PO_4, in a minimum amount to obtain a ratio of C:N:P almost equal to 100:5:1 [35] for heterotrophic bacteria metabolism.

2.4. Analytical procedures

The COD, total suspended solids (TSS), volatile suspended solids (VSS) concentrations and the sludge volume index (SVI) were determined according to the Standard Methods [36]. All analysis were performed in triplicates. COD_T was directly measured in the sample, while the COD_S from the sample filtered through 0.45 µm pore size filters. The morphology and size distribution of granules were measured regularly by using an Image Analysis procedure [37] with a stereomicroscope. Biomass density, in terms of gVSS per litre of granules, was determined with dextran blue and following the methodology proposed by Beun et al. (2002) [34]. Granulation rate was evaluated as the percentage of particles with a diameter over 600 µm, according to Liu et al., (2012) [38]. pH and dissolved oxygen (DO) measurements were performed by means of portable on-line sensors. The total Extracellular Polymeric Substances were expressed as the sum of bound EPSs and soluble microbial products (SMPs), as protein and polysaccharide fractions. Then, the EPS content was referred to the VSS concentration. The SMPs were obtained by centrifugation at 5000 rpm for 5 min while the bound EPSs were extracted by a thermal extraction method [39]. The polysaccharides were determined according to the phenol–sulphuric acid method with glucose as standard [40], while the proteins were determined by the Lowry method with bovine serum albumin (BSA) as standard [41]. The samples for EPSs analysis were taken twice in a reaction cycle. In particular, the first sample was taken at the end of the feast phase, when most of the substrate was oxidized, while the second sample was taken at the end of the famine phase. In order to establish the time when the substrate was consumed during the feast phase, experimental measurements of pH and COD_S were assessed during the operational cycle. Although nitrogen removal was not the objective of this study, at the beginning of feast phase the high amount of exogenous COD_S represented the e-donor for the denitrification of the NOx (nitrites and nitrates) produced in the process, thus resulting in the overall simultaneous nitrification–denitrification (SND) mechanism. Therefore, this meant that the pH of the mixed liquor, that was the expression of the balance between the pH increase due to denitrification and the pH decrease due to the nitrification, globally increased thus indicating a prevalence of denitrification process. When the pH signal reached a maximum, the exogenous CODs was almost all consumed (data confirmed by experimental sampling and CODs analysis at the instant when pH was at its maximum value), thus establishing the end of feast phase and the beginning of famine phase.

Table 2
Operational condition for R1 and R2.

Reactor	Phase	Days of operation	OLR (kg CODm^{-3}d^{-1})	Salinity (gNaCl·L^{-1})	TPH (mg·L^{-1})	Slop WW (v/v)
R1	I^a	0–30	1.7 ± 0.1	37.8 ± 1.2	15 ± 6	10%
	II^a	30–60	1.7 ± 0.1	37.8 ± 1.2	30 ± 3	20%
	III^a	60–90	1.6 ± 0.1	37.8 ± 1.2	60 ± 4	40%
	IV^a	90–120	1.7 ± 0.1	37.8 ± 1.2	105 ± 2	70%
	V^a	120–150	1.6 ± 0.1	37.8 ± 1.2	151 ± 3	100%
R2	I^b	0–30	1.6 ± 0.1	1.8 ± 0.7	15 ± 6	10%
	II^b	30–60	1.7 ± 0.1	4.9 ± 0.7	30 ± 3	20%
	III^b	60–90	1.7 ± 0.1	11.6 ± 0.3	60 ± 4	40%
	IV^b	90–120	1.6 ± 0.1	24.3 ± 2.7	105 ± 2	70%
	V^b	120–150	1.6 ± 0.1	37.8 ± 1.2	151 ± 3	100%

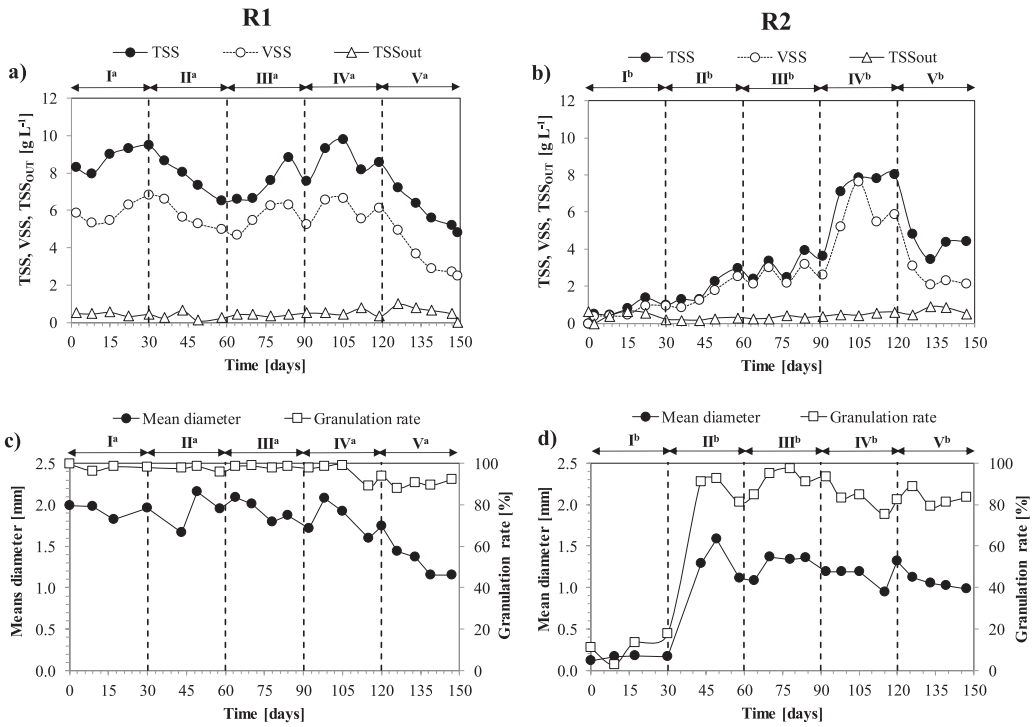

Fig. 1. Total suspended solids, volatile suspended solids in the reactor and total suspended solids in the effluent in R1 (a) and in R2 (b). Average diameter and granulation rate in R1 (c) and in R2 (d).

2.5. Total petroleum hydrocarbons (TPH) extraction and analysis

TPHs were extracted from liquid phase by means of a separatory funnel Liquid-Liquid Extraction (LLE) according to the standardized method EPA 3510c [42]. Regarding to the solid phase represented by aerobic granules, TPHs were extracted by means of a Pressurized Fluid Extraction (PFE) (with a Speed Extractor E-916, Buchi®) according to the method EPA 3545a [43] for soil and sludge. This method was applied to granular biomass to evaluate the bio-adsorbed fraction of hydrocarbons. Subsequently, the extracts were analysed with a Gas Chromatograph equipped with a Flame Ionization Detector (GC-FID Agilent® 6890 N).

In both the cases, the extract was injected into the GC-FID system, through an auto-sampler, and analysed according to the method EPA 8015c [43] to analyse the hydrocarbons in the range of numbers of carbons C10-C40, thus including the diesel range organics (DRO) and the oil range organics (ORO), after calibration. The chromatographic conditions were: carrier gas (helium) flow rate: 5–7 mL·min^{-1}; makeup gas (helium) flow rate: 30 mL·min^{-1}; injector temperature: 200 °C; detector temperature: 340 °C; initial temperature: 45 °C, hold for 3 min.

For the analysis of DRO, the areas of all peaks eluting between C10 and C28 were summed. These areas were generated by projecting a horizontal baseline between the retention times of C10 and C28.

3. Results and discussion

3.1. Physical properties of granules

Fig. 1a and b show the TSS and VSS in the reactors and the TSS in the effluent for R1 and R2, respectively.

In R1 (Fig. 1a), when slop was dosed with a concentration of hydrocarbons next to 15 mgTPH·L^{-1} (Phase I^a), the mature salt-adapted granules responded with an initial slight decrease of biomass concentration from 5.9 to 5.3 gVSS·L^{-1} due to a partial biological inhibition of microorganisms in presence of the new recalcitrant substrate. Then, an increase of volatile solids concentration up to 6.8 gVSS·L^{-1} denoted the adaptation of granules to the new environmental conditions. However, in Phase II^a the further increase of hydrocarbons concentration from 15 to 30 mgTPH·L^{-1} implied a rapid decrease of biomass concentration from 6.6 to 4.9 gVSS·L^{-1}, highlighting a strong inhibition of microorganisms subjected to the increasing dosage of recalcitrant slop. During Phase III^a and IV^a the trend of solids was fluctuating and reached a concentration of 5.5 gVSS·L^{-1} at the end of Phase IV^a (105 mgTPH·L^{-1}). Finally, a strongly decreasing trend down to 2.5 gVSS·L^{-1} indicated a severe biological inhibition of biomass when only slop was fed to the reactor during Phase V^a (151 mgTPH·L^{-1}). During this phase, the continuous decreasing trend suggested a negative net-growth due to the bacteria disappearance and death. Moreover, in this phase a peak of effluent solids concentration next to 1 gTSS·L^{-1} was observed because of a partial degranulation. Throughout all the experimental period, the VSS/TSS ratio in R1 was most of the time below 70% due to the high portion of inorganic salts that deposited into the salt-adapted granules during the saline cultivation discussed in a previous research [4]. Then, in the last phase, this ratio decreased down to 50% due to the decrease of the organic fraction of biomass that underwent a strong inhibition.

Observing the evolution of solids in R2 (Fig. 1b), since in this reactor granules were cultivated from flocculent biomass, with a simultaneous increase of salinity and hydrocarbons, the granulation process implied a gradual increase of biomass concentration from 0.4 gVSS·L^{-1} at the beginning of Phase I^b (1.8 gNaCl·L^{-1} and 15 mgTPH·L^{-1}), up to 3.2 gVSS·L^{-1} at the end of Phase III^b (11.6 gNaCl·L^{-1} and 60 mgTPH·L^{-1}). Therefore, the simultaneous increase of salinity and hydrocarbons did not involve any obstacle to granulation of biomass. Then, in Phase IV^b (24.3 gNaCl·L^{-1} and 105 mgTPH·L^{-1}) a sudden increase of VSS till about 7.6 g·L^{-1} suggested a phenomenon of bio-adsorption of particulate hydrocarbons of the feeding into the granules porosity, as discussed in the following. Then, in Phase V^b (37.8 gNaCl·L^{-1} and 151 mgTPH·L^{-1}) the decrease and stabilization of solids down to 2.3 gVSS·L^{-1}, highlighted a probable biodegradation of bio-adsorbed fraction of hydrocarbons. It could be stated that during the last phase where only slops were fed to the

reactors, in R2 the solids concentration had stabilized around the stationary value of 2.3 gVSS·L^{-1} and the stationary condition implied a biological activity of microorganisms with a simultaneous growth and decay of bacteria. Since the sole substrate fed to the reactors in this last phase was the real slop, it could be supposed that in R2 biomass growth occurred over hydrocarbons contained in slop wastewater that constituted the sole electron-donor. This did not occur in R1, where a continuous decrease of VSS was observed during the last phase, confirming a biological inhibition of biomass in that case.

The VSS/TSS ratio in R2 was always higher than in R1 (over 70%) and registered a decreasing trend because granules were cultivated with a step-wise increase of both salt and hydrocarbons. Therefore, the inorganic salts precipitated inside the granules porosity during the cultivation and granulation process, as reported by Campo et al. (2018) [4], thus increasing the inorganic fraction of solids. From a global perspective, it is possible to see a convergence of both TSS and VSS to values of 4.4–4.8 gTSS·L^{-1} and 2.2–2.5 gVSS·L^{-1} in the last phases V^a and V^b for R1 and R2, respectively.

Regarding to the granules dimensions, in R1 the mean diameter of granules decreased from about 2 mm down to 1.15 mm throughout all the experimental period (Fig. 1c). Moreover, the reduction of the granulation rate from 98% to 89% confirmed that a partial degranulation occurred, especially in Phase V^a when only slop was fed to the reactors. In R2 (Fig. 1d), a sudden aggregation of flocculent biomass to granules was observed, straddling the Phase I^b and the Phase II^b, confirmed by a rapid increase of granules mean diameter from 0.16 mm to 1.28 mm and a simultaneous jump of granulation rate from 17% up to 91%. The increase of salt and hydrocarbons concentration from Phase I^b to Phase II^b enhanced the aggregation of microorganisms as a mechanism of self-defence in critical environmental conditions [4,44]. Then, a slight regular decrease from 1.58 mm down to 1 mm was observed from Phase II^b to Phase V^b and, during the same period, the granulation rate fluctuated around the mean value of 85%. Also in this case, a convergence of the mean diameter of granules was observed for both R1 and R2, denoting similar physical properties and behaviour of both kinds of granular biomass. However, the slight low mean diameter in R2 (1 mm vs 1.15 mm in R1) conferred a higher specific surface area to granules, thus enhancing the bio-adsorption capacity of the granular sludge [45,46].

Table 3 includes other granules features like the SVI_5 and granules density. In R1, the salt-adapted mature granules showed a quite stable SVI_5 around 11.8 mL·g^{-1} (average value throughout the experimental period), highlighting excellent settling properties. Moreover, the density progressively increased from 75 to 97 gTSS·L^{-1} mainly due to the adsorption of hydrocarbons. In R2, the granulation process implied a rapid improvement of the settling abilities of granules and the SVI_5 dropped from 60 to 20 mL·g^{-1}, from Phase I^b to Phase II^b. Then, it reached 10 mL·g^{-1} at the end of the experimentation. Similarly, the density suddenly increased from 32 to 65 gTSS·L^{-1} from Phase I^b to Phase II^b, due to the bio-aggregation of microorganisms. Then, a peak of 110 gTSS·L^{-1} in Phase IV^b suggested an important adsorption of hydrocarbons into the granules porosity, thus becoming heavier and denser. Finally, the density of granules recovered its magnitude next to 72 gTSS·L^{-1} in Phase V^b, due to a probable biodegradation of the adsorbed hydrocarbons.

Table 3
SVI_5 and density of aerobic granules in R1 and R2 (average values).

Reactor	Phase	SVI_5 (mL·g^{-1})	Density (gTSS·L^{-1})
R1	I^a	11 ± 2	75 ± 1
	II^a	14 ± 3	78 ± 3
	III^a	15 ± 1	81 ± 2
	IV^a	11 ± 2	80 ± 4
	V^a	8 ± 1	97 ± 3
R2	I^b	60 ± 5	32 ± 2
	II^b	20 ± 3	65 ± 4
	III^b	22 ± 4	67 ± 2
	IV^b	23 ± 1	110 ± 5
	V^b	10 ± 1	72 ± 3

Interesting considerations can be drawn from particle size distribution (PSD) of granules in R1 and R2 (Fig. S3).

In R1, a slight and gradual decrease of mean dimensions did not involve a great variability of the density distribution of particle sizes of granules. This implied that granules were distributed around the mean value, throughout the experimental period. Just a larger density distribution was observed in Phase V^a, where granules reduced their dimensions and underwent a partial degranulation, as discussed previously.

A completely different behaviour was observed in R2. Firstly, the granulation process implied a sensible variation in PSD from Phase I^b to Phase II^b due to the bio-aggregation of microorganisms. Moreover, compared to R1, in R2 the density distribution of particle sizes of granules was characterized by multimodal shape and some values were dispersed from the average value. This implied a granular system with a more heterogeneous distribution of granules dimensions. Throughout all the experimental period mean granules dimensions of R1 were higher than R2. Therefore, the higher specific external surface area of granules in R2, not considering the internal porosity, offered a higher adsorbent external surface thus improving the initial bio-adsorption mechanism represented by adhesion onto the external surface of aerobic granules.

The morphology and the appearance of granules in R1 and R2 are shown in Fig. 2. Observing the R1 granules (Fig. 2a,c,e,g and i), it is possible to see how they maintained their round-shaped morphology gained in the previous saline cultivation [4] throughout all the experimental period, although their dimensions gradually decreased. Moreover, a gradual change in their colour appearance from yellow in Phase I^a, to reddish in Phase III^a and, finally, to brown in Phase V^a was observed. This gradual change in colour could be likely due to both the progressive adsorption of hydrocarbons inside the granules structure [46] and to a change in microbial composition in the biofilm.

In R2 (Fig. 2b,d,f,h and j), the granulation process with the industrial slop wastewater, with a simultaneous increase of salinity and hydrocarbons, implied the development of granules with a wedge-shape and an irregular morphology. Especially in Phase IV^b (Fig. 2h), it is possible to see a great variability of granules morphology and dimensions, as confirmed by the PSD. Moreover, the bio-adsorbed hydrocarbons during the granulation process, induced a reddish-brown appearance starting from the Phase II^b, until the end of the experimentation.

3.2. Extracellular polymeric substances (EPS)

EPS have a crucial role in granules formation and in bio-adsorption properties [45–48]. Moreover, the production of extracellular polymeric substances is an indirect indicator of the biological activity of microorganisms. Fig. 3 shows the SMPs and the EPS expressed as proteins (PN) and polysaccharides (PS). Both in R1 (Fig. 3a) and in R2 (Fig. 3b), a gradual increase of the protein fraction of SMP was observed from Phases I^a–I^b to Phases IV^a–IV^b, up to a value of 15–20 mg·gVSS^{-1} for both the reactors. Then, a sudden increase up to 74 and 95 mg·gVSS^{-1}, for R1 and R2 respectively, was registered in Phases V^a–V^b. The most probable explanation for this behaviour is that, when microorganisms were subjected to stressful environmental conditions, they reacted by increasing their microbiological activity in order to face the stress of the environmental condition. In this case the microorganisms released proteins SMP, probably from cell-lysis, in response to the presence of hydrocarbons (R1) and both salinity and hydrocarbons (R2). This effect was more evident in the last phases, when only real slop was fed to the reactors.

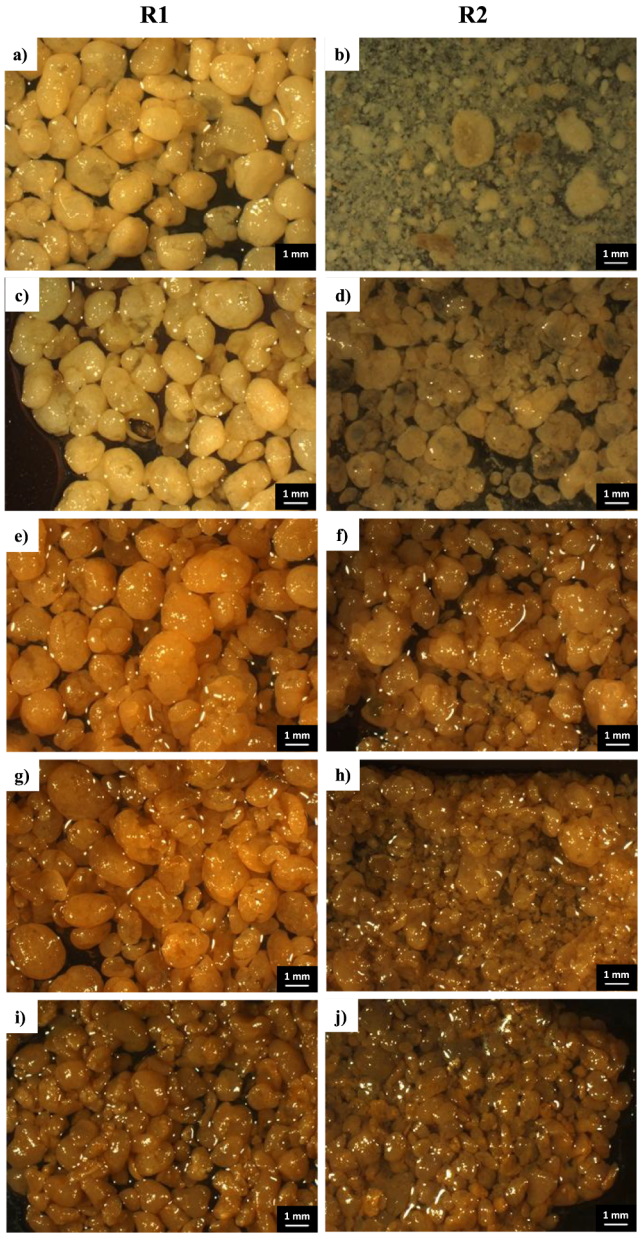

Fig. 2. Aerobic granules in R1 (a,c,e,g,i) and R2 (b,d,f,h,j) (7X magnification): Phases I^a, I^b – day 9 (a,b); Phases II^a, II^b – day 58 (c,d); Phases III^a, III^b – day 84 (e,f); Phases IV^a, IV^b – day 115 (g,h); Phases V^a, V^b – day 147 (i,j).

Observing the polysaccharides fraction of SMP, in R1 the evolution was quite constant and regular, around the low value of 5 mg·gVSS^{-1} (Fig. 3c). The mature salt-adapted granules were more robust and able to face the recalcitrant substrate, thus producing a low amount of SMP. In R2 the trend of polysaccharides SMP (Fig. 3d) was quite different due to both the granulation process of the flocculent biomass, especially during the Phases I^b and II^b, and the simultaneous adaptation to salt and hydrocarbons. Therefore, in the first two phases the microorganisms modified their biological pathways during the granulation process in presence of the recalcitrant substrate, producing a higher concentration of polysaccharides SMP next to 25 mg·gVSS^{-1} (Phase I^b) and 13 mg·gVSS^{-1} (Phase II^b). Then, when mature granules were formed, a gradual decrease of the concentration of polysaccharides SMP was observed.

Looking at the EPS bound to the bacterial cells, after the heating method extraction granules of both the reactors appeared almost totally disaggregated, denoting an efficient extraction protocol. Moreover, in R1 (Fig. 3e) the protein fraction of EPS bound was lower (around 40 mg·gVSS^{-1}) than in R2 (Fig. 3f) (around 75 mg·gVSS^{-1}). However, in R1 a peak in Phase II^a was observed due to the increase of hydrocarbons concentration from 15 to 30 mgTPH·L^{-1}. Since proteins (PN) are well-known to have a structural function in biological systems such as AGS [4,47], the higher values of bound PN in granules of R2 could indicate that granules of R2 had a more stable structure than granules of R1.

By analysing the polysaccharides fraction of bound EPS, in R1 (Fig. 3g) the trend was quite stable and constant, as observed previously for SMP, around the average value of 11 mg·gVSS^{-1}, because in R1 granules were mature and pre-cultivated to salinity. In R2 (Fig. 3h), during the first Phases I^b and II^b, it is possible to see a higher value of polysaccharides EPS, around the average value of 23 mg·gVSS^{-1}. During the bio-aggregation process, microorganisms produced more polysaccharides that are well-known to have jelly-like [47,49] and sticky properties, thus enhancing the adhesion and the granules development. Then, when mature granules were formed, the bound polysaccharides fraction decreased to an average value of 8 mg·gVSS^{-1}.

The lower specific values of PN and PS of bound EPS compared to other works [30,50,51], is likely due to the different substrate fed to the reactor and to the higher concentrations of TPHs in this study. Since almost all EPS extraction methods are not able to extract all the EPSs from biomass [47], the real purpose of this study was not to operate a completely-full extraction of EPSs, but to perform a relative comparison between EPSs extracted throughout all the experimental period, given that the extraction method applied was always the same.

. Moreover, comparing the EPS results in feast and famine phases, as observed in a previous research [4], also in this case the concentrations in feast phase were globally lower than the concentrations in famine phase, for some days of operation. Also in this experience, a possible explaination of this occurrence could be related to microorganisms' degradation of bound EPS during feast phase in a higher extent respect to the famine phase, in order to gain more energy to face the osmotic stress caused by the salinity and the toxic stress caused by the hydrocarbons.

The higher the value of PN/PS, the greater the stability and the robustness of the granular sludge, since the bound proteins confer structural strength and stability to granular sludge [4,47]. Fig. S4a,b show the PN/PS ratio in R1 and in R2 evaluated both in feast and famine phase. In R1, the PN/PS ratio was almost constant throughout the experimental period, around an average value of 4.7 mg·gVSS^{-1}. The mature salt-adapted granules maintained their structural strength by preserving the PN/PS ratio around an average value of 4.7, when subjected to a gradual increase of hydrocarbons concentration. In R2, the trend of PN/PS ratio was completely different. During the Phase I^b the PN/PS ratio of flocculent biomass was next to 2.3. Then, when bio-aggregation occurred in Phase II^b, a sudden increase of PN/PS ratio up to 5.6 was registered, denoting the formation of the structure of granules with proteins chains. Respect to the PN/PS ratios obtained in other studies [50,51], the results achieved in this experimental work were consistent and similar. Although the specific values of PN and PS were different, PN/PS ratio was comparable thus denoting a similar behaviour of microorganisms forming the aerobic granules that maintained almost the same proportion of proteins and polysaccharides in their EPS matrix.

Comparing the PN/PS ratio with the Fig. 3h, it is possible to see how, during the Phase I^b polysaccharides were produced in higher quantity than in the following phases. This favoured the adhesion of single flocs in a gelatinous matrix favouring the bio-aggregation and, consequently, the PN/PS ratio was quite low. Then, when the bio-aggregation of the microorganisms began, the PS production decreased and the PN production increased (Fig. 3b), thus resulting in a global increase of the PN/PS ratio. In Phase III^b the PN/PS ratio was almost constant around the average value of 9.2, suggesting the maturation of the formed granules, that was maintained also in Phase IV^b with a PN/

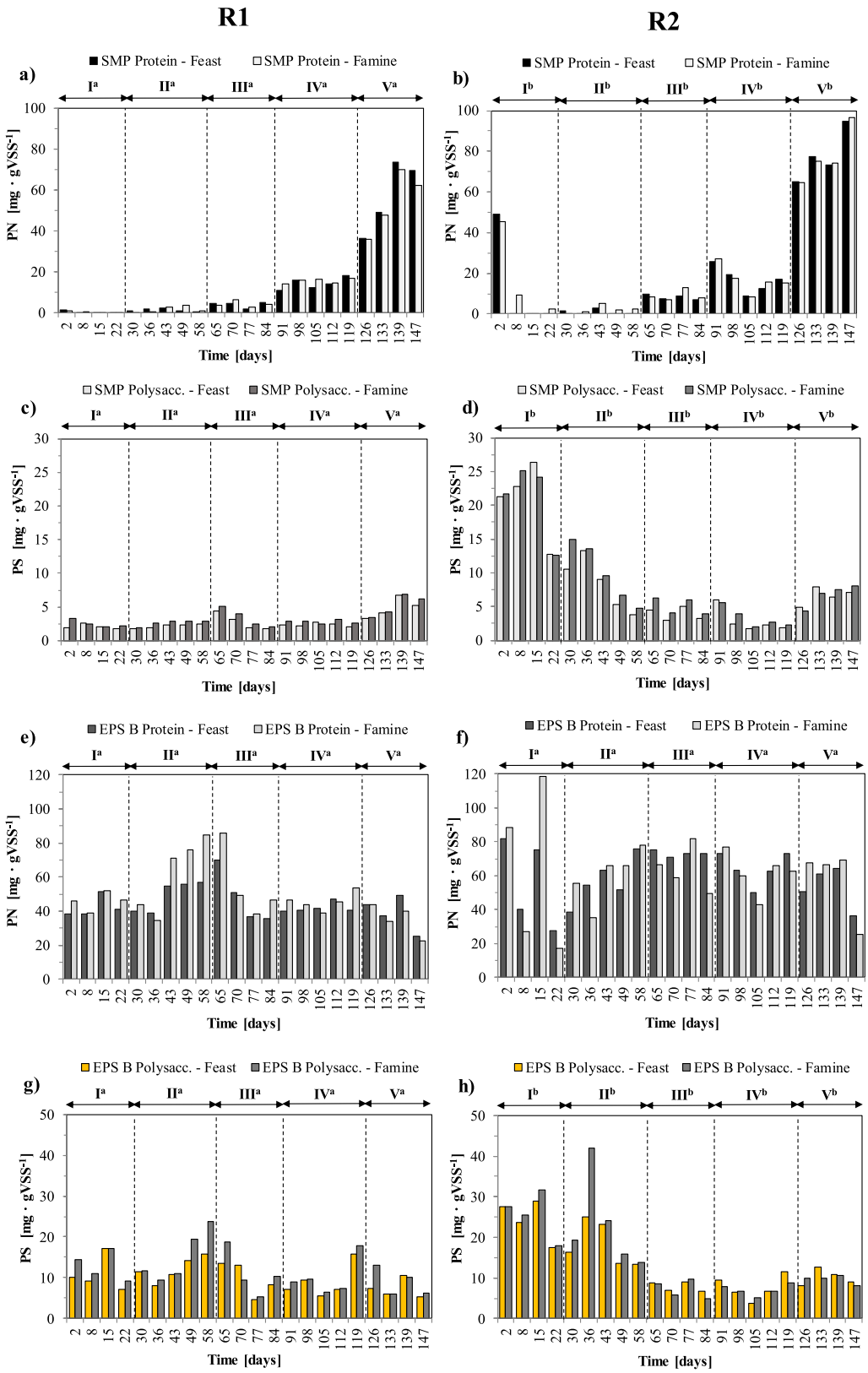

Fig. 3. Soluble microbial products (SMP) and extracellular polymeric substances (EPS) bound to the microorganisms, in protein (PN) and polysaccharides (PS) form in R1 (a,c,e,g) and R2 (b,d,f,h).

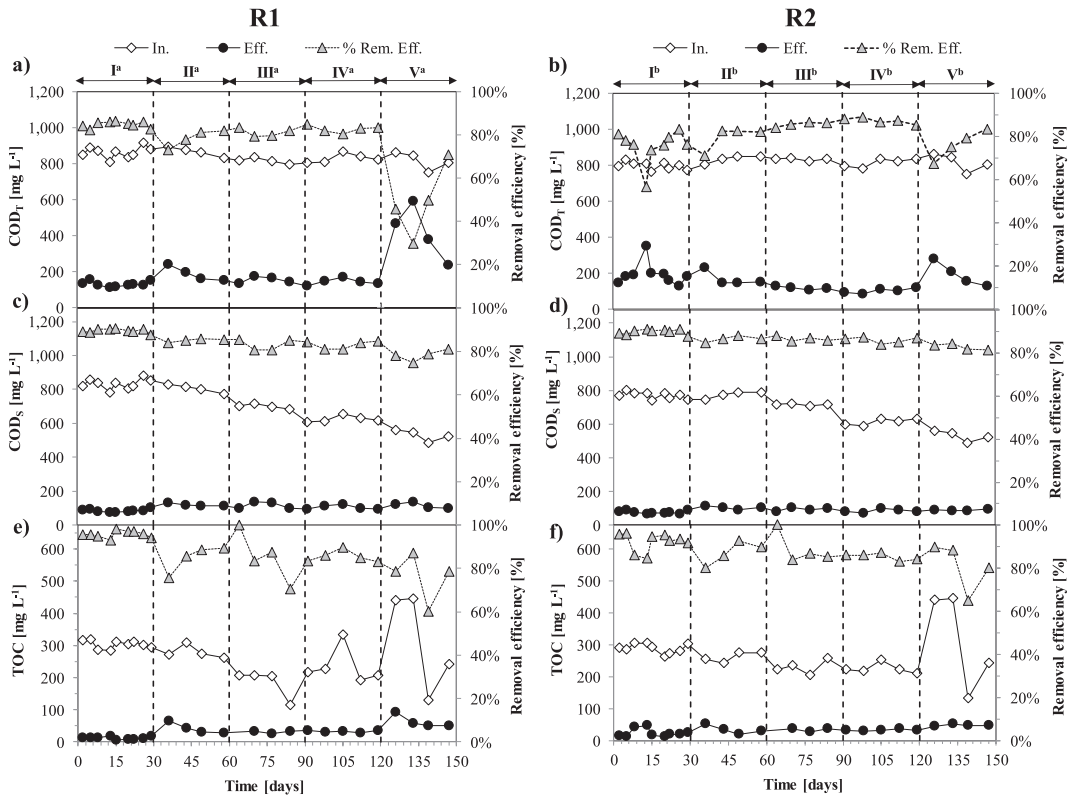

Fig. 4. Organic matter removal in terms of COD_T, COD_S and TOC for R1 (a,c,e) and R2 (b,d,f).

PS next to 9.1. However, in the last Phase V^b the decrease of PN/PS ratio down to 3.6 when only slop was fed to the reactor, implied a slight loss of stability of granules that involved a partial degranulation, as discussed previously.

3.3. Organic matter removal

In R1, the COD_T (Fig. 4a) was removed with an efficiency higher than 80% until the end of Phase IV^a. Then, when only slop wastewater was fed to the reactor (Phase V^a), the removal efficiency collapsed down to 30%, due to two main probable causes: a strong biological inhibition of microorganisms when the sole substrate was represented by the hydrocarbons of the slop wastewater; a physical desorption phenomenon of particulate hydrocarbons from saturated granules. However, after the day 133 a recovery of COD_T removal efficiency up to 70% was observed and, also in this case, this could be due to both a biological adaptation and a physical bio-adsorption in the regenerated sites of aerobic granules.

In R2 (Fig. 4b), once granules were formed (starting from Phase II^b), the COD_T removal efficiency was always higher than 80%. When only slop was fed to the reactor (Phase V^b), a smaller decrease of the removal efficiency down to 67%, was observed, compared to R1. After the day 126, a progressive recovery of removal efficiency was observed, up to 84%. Also, in this case, this trend in the last phase could be due to both an initial biological inhibition and a physical desorption, followed by a biological adaptation and physical bio-adsorption, in a higher extent respect to R1. However, to better understand what effect between biological or physical occurred in a prevailing mode, further analysis on the solid phase (i.e. aerobic granules) will be discussed in the following. Regarding to the CODs, the removal efficiencies were higher than 80% for both the reactors throughout all the experimental period (Fig. 4c and d). Moreover, it should be stressed that the decreasing trend of the influent COD_S was due to the gradual increase of slop dosage, that notoriously contains particulate matter. Finally, observing the TOC removal efficiencies (Fig. 4e and f), values higher than 80% were globally registered for both reactors, except for some spots the days 36 (Phase II^a), 84 (Phase III^a) and 139 (Phase V^a) for R1, where TOC removal efficiencies of 76%, 70% and 60% were registered respectively, and during the day 139 (Phase V^b) for R2, where a TOC removal efficiency of 60% was obtained.

3.4. TPH removal

In order to provide a more specific insight about the organic matter removal, the effect of aerobic granular biomass towards the TPHs removal was assessed in both the reactors. To investigate if also a biological removal of TPHs occurred, as an additional mechanism to bio-adsorption phenomena into the porosity of granular sludge, TPHs concentration was analysed in the influent, in the effluent and adsorbed in the solid phase of granules, for R1 and R2 (Fig. 5).

In R1, the TPH was removed in liquid phase (Fig. 5a) with a removal efficiency higher than 90%, until the Phase III^a (60 $mgTPH \cdot L^{-1}$). At the same time, only a negligible amount was found adsorbed into the granules (Fig. 5c), thus suggesting that until a concentration of hydrocarbons up to 60 $mgTPH \cdot L^{-1}$, the microorganisms forming the aerobic granules were probably able to biodegrade the hydrocarbons in liquid phase and adsorbed into the granules. These removal efficiencies continued in Phase IV^a (105 $mgTPH \cdot L^{-1}$). Then, in Phase V^a (151 $mgTPH \cdot L^{-1}$) when only slops were fed to the reactors, the effluent concentrations of TPH exceeded the influent concentrations, the days 126 and 133. This unusual observation is explainable with an initial strong inhibition of biomass when the recalcitrant substrate was the sole carbon source fed to the system, and with a physical desorption and release of hydrocarbons from saturated and inhibited aerobic granules. Then, a partial recovery of the TPH removal efficiency up to 36% the day 147 combined with a new increase of the adsorbed fraction, suggested that when only slop was fed to R1, the main mechanism for TPH removal was a physical bio-adsorption phenomenon. Moreover, bearing

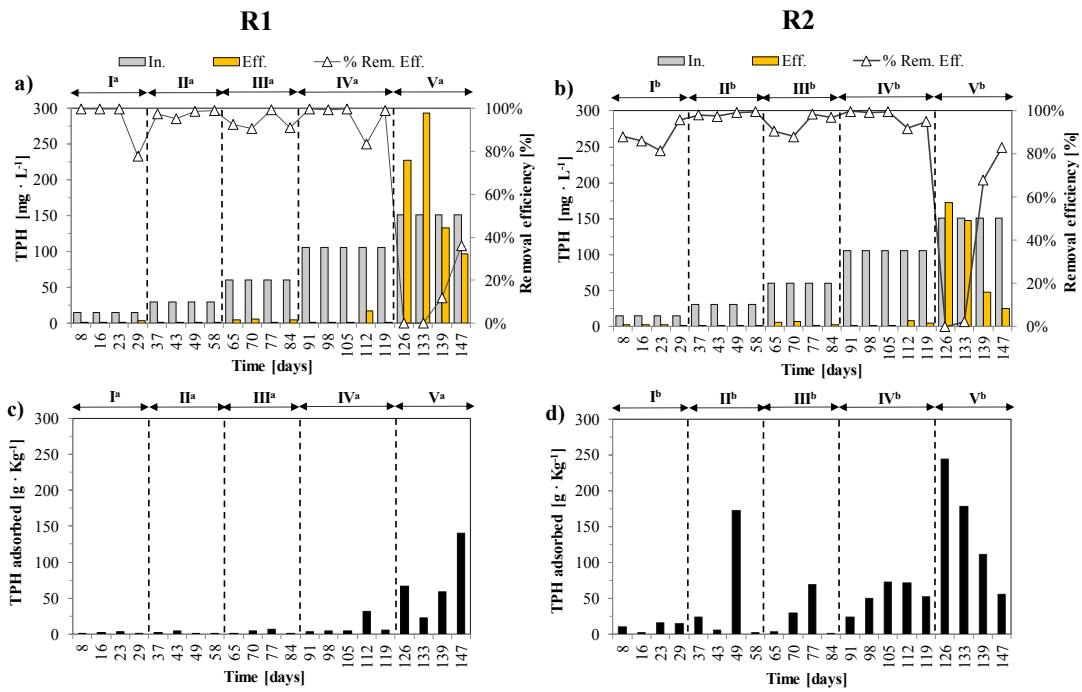

Fig. 5. TPH in the influent, in the effluent and removal efficiencies in R1 (a) and in R2 (b); TPH adsorbed on granules in R1 (c) and R2 (d); correlations between TPH adsorbed into the granules surface and the PN/PS ratio, in R1 (e) and in R2 (f).

in mind the above, since low removal efficiencies were registered when only slop was fed to the reactor, it could be stated that the high TPHs removal efficiencies registered in the previous phases were mainly due to a co-metabolic biological pathway, as observed by Zhang et al. (2011) [13]. In fact, the mixture of sodium acetate and slop to maintain an almost constant OLR fed to the reactors until the Phases IV^a and IV^b, as described in the previous Section 3.3, may had induced the co-metabolism mechanism. As well-known from the scientific literature, co-metabolism is defined as the simultaneous degradation of two compounds, in which the degradation of the second compound (the non-growth substrate and in this case the hydrocarbons representing the recalcitrant fraction of slop wastewater) depends on the presence of the first compound (the growth substrate and in this case the sodium acetate). Moreover, co-metabolism occurs by the action of enzymes that were produced by the microorganisms primarily for some other purpose, such as the degradation of the primary growth substrate [30,52]. Therefore, during the first four phases, the metabolism of sodium acetate could have provided sufficient carbon and energy sources for microbial growth, and it could have induced the production of corresponding degrading enzymes to degrade the recalcitrant hydrocarbons, thus expanding the selective range of carbon and energy sources for microorganisms.

By observing the R2 data in liquid phase (Fig. 5b), except for the Phase I^b in which flocculent sludge was still present, until the Phase IV^b the TPH removal efficiency was higher than 90% probably due to the combined effect of co-metabolism and bio-adsorption (Fig. 5d). In particular, during Phase IV^b a gradual accumulation of TPH adsorbed into the granules was observed, suggesting that in this phase the physical bio-adsorption was the main mechanism, as outlined previously.

Then, in Phase V^b, a slight outflow of TPH higher than the influent was observed the day 126, due to both an initial biological inhibition when TPH concentration increased from 105 to 151 mgTPH·L^{-1} and to a probable saturation-desorption of hydrocarbons from aerobic granules. Then, from the day 133, a gradual recovery of TPH removal in liquid phase was observed up to 83%. Observing the trend and the concentrations of TPH adsorbed in the solid phase of granules in Phase V^b (Fig. 5d), it is possible to see a progressive decrease of the adsorbed hydrocarbons that, combined with the simultaneous removal in liquid phase, suggested a biological adaptation of microorganisms forming granules to TPHs. Therefore, it is possible to assert that in this phase, hydrolysis and biodegradation occurred. By comparing the two applied strategies in R1 and R2, it is possible to globally state that aerobic granular sludge is an effective technology able to remove recalcitrant substrates. However, when using mature granules as seed sludge (R1) to treat the industrial wastewater the biological adaptation is difficult since microorganisms were already aggregated in a compact and dense structure and protect themselves from any recalcitrant/toxic external compound, although it is dosed with a step-wise increase strategy. Therefore, in this case, the main removal mechanism is represented by physical bio-adsorption. On the other hand, when granules were directly cultivated with the industrial wastewater containing recalcitrant compounds like hydrocarbons (R2), both the granulation process involving EPS production and bio-aggregation, combined with the step-wise increase of industrial wastewater dosage, favoured the development of aerobic granules based on the growth of proper specialized microorganisms able to hydrolyse and biodegrade the recalcitrant substrate. In other words, the cultivation and the granulation of aerobic granules with industrial wastewater containing recalcitrant substrate acts as a physical and biological selector, thus favouring the development of robust and mature aerobic granules and, at the same time, promoting the growth of specialized biomass composed by strong and TPH-adapted microorganisms. However, microbiological analysis were out of the scope of this work. An interesting correlation was found between the concentration of TPH adsorbed into the granules surface and the PN/PS ratio (Fig. S4c,d,e,f,g) that is an indicator of structural strength of granules since proteins have bulking-agent properties, thus conferring robustness to aerobic granules. Good correlations between the TPH adsorbed into the granules surface and the PN/PS ratio in R1 were observed during Phase II (R^2 = 0.86) and Phase III (R^2 = 0.72), but they decreased as the concentration of hydrocarbons in the reactor increased. In R1, mature salt adapted granules did not produce a high concentration of EPS, as discussed previously. Therefore, the higher the saturation of the aerobic granules, the weaker is the correlation between TPH and PN/PS, since the increased TPH adsorbed on granules

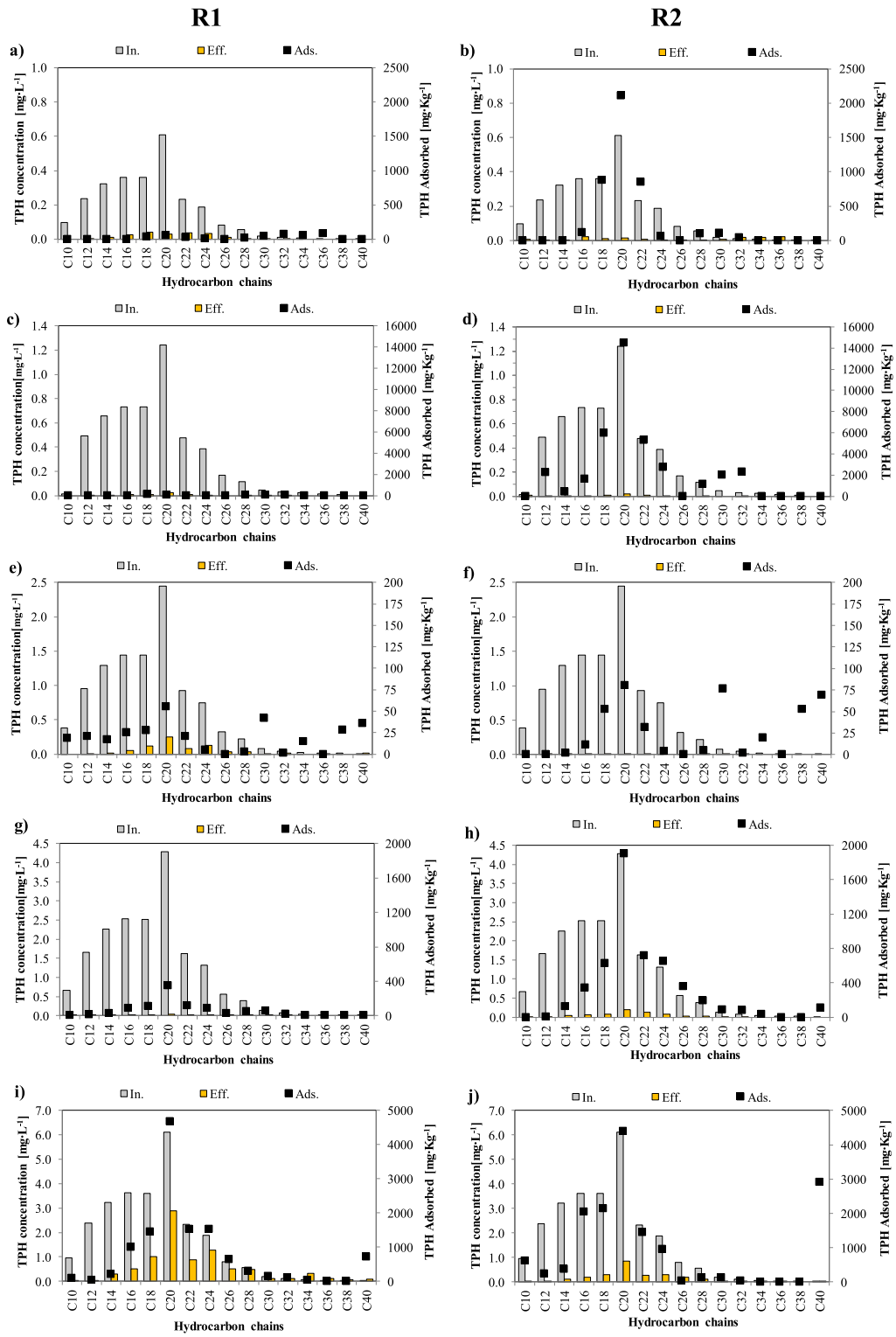

Fig. 6. Hydrocarbons speciation in the range C10–C40, in the influent, in the effluent and adsorbed on granules in R1 (a,c,e,g,i) and R2 (b,d,f,h,j): Phases I^a, I^b – day 29 (a and b), Phases II^a, II^b – day 49 (c and d), Phases III^a, III^b – day 84 (e and f), Phases IV^a, IV^b – day 119 (g and h), Phases V^a, V^b – day 147 (I and j).

surface limits the driving force represented by the gradient of TPH concentration between the bulk and the granules. In R2 the correlation was relatively high during the granulation process. As discussed previously, during the first two Phases in R2 the granulation process involved a higher production of EPS, enhancing the microbial aggregation. This favoured a simultaneous adsorption of TPH with an increasing correlation between TPH adsorbed and PN/PS from Phase II^b (R^2 = 0.58) to Phase IV^b (R^2 = 0.73). Then in Phase V^b, granules saturation and lower EPS production implied a lower correlation between adsorbed TPH and PN/PS (R^2 = 0.61). Then, the regeneration of some adsorbent sites on granule surface, probably due to a mechanism of TPH biodegradation, favoured a gradual increase of the correlation between hydrocarbons and PN/PS, suggesting an active mechanism of bio-adsorption and biodegradation. Moreover, globally higher concentrations

of adsorbed TPHs were registered for R2 (up to 244 gTPH·kg^{-1}), compared to R1 (up to 140 gTPH·kg^{-1}) due to the higher adsorption capacities of R2 than R1 as a consequence of the higher EPS production during the granulation phase and the higher specific surface area linked to the lower granules mean dimensions.

In order to better understand the phenomenon, a hydrocarbons' speciation focusing on the concentrations of single hydrocarbon chain in the range C10-C40 in the influent, in the effluent and adsorbed into the granules was assessed in R1 and R2, respectively. The analyses were performed at the end of each Phase. By observing the hydrocarbons speciation during Phase I^a in R1 (Fig. 6a), and during Phase I^b in R2 (Fig. 6b), it is possible to see how the adsorption phenomenon was the prevailing mechanism during Phases I^a-I^b, especially in R2. More specifically, in R1 a lower adsorbed concentration of hydrocarbons was observed, compared to R2, since stable and mature salt-adapted granules produced less EPS, thus contributing to a less adsorption capacity. In R2, the higher adsorbed TPH concentration can be associated to the higher EPS production that occurred during the granulation process, as discussed previously. Moreover, the higher specific surface area due to the smaller dimensions of the aggregates in R2 registered in this phase, exerted a higher bio-adsorbent capacity.

Observing the hydrocarbons speciation during Phase II^a in R1 (Fig. 6c), and during Phase II^b in R2 (Fig. 6d), no sensible variation in the behaviour of R1 was observed, respect to the previous phase. On the other hand, in R2 a considerable increase of the adsorption fraction was observed, compared to the previous phase. As discussed previously, during the Phase II^b the bio-aggregation occurred, and this favoured the bio-adsorption mainly due to the high EPS production. During Phase III^a in R1 (Fig. 6e), and during Phase III^b in R2 (Fig. 6f), the lower hydrocarbons adsorbed on granules and the lower hydrocarbons concentration in the effluents of both the reactors, suggested a probable adaptation of microorganisms at the influent hydrocarbons concentration. As discussed previously, as the influent was composed of a mixture of synthetic and real wastewater, it is possible that a co-metabolic pathway occurred in both the reactors, thus enhancing the hydrocarbons biological removal.

During the Phase IV^a in R1 (Fig. 6g), and during the Phase IV^b in R2 (Fig. 6h), the bio-adsorbed fraction of hydrocarbons increased in both the reactors, with a greater extent in R2. Both the systems were not able to biologically remove the increase load of TPH and underwent a biological inhibition when the slop dosage increased. Therefore, granules responded with a sensible increase of the adsorbed fraction of hydrocarbons. This confirmed the great peculiarity of granules whereby, when the biodegradation is affected, the physical bio-adsorption helps the global removal of TPH. This mechanism implies an increase of the contact time between the recalcitrant compounds and the microorganisms, thus enhancing the possibility to hydrolyse and biodegrade also the less biodegradable substrates.

Finally, observing the data of Phase V^a in R1 (Fig. 6i), it is possible to note that, when only real slop was fed to the reactors, a considerable increase of adsorbed fraction occurred in R1, compared to the previous phase. Moreover, higher concentrations of hydrocarbons in the effluent were registered, highlighting a probable desorption phenomenon of TPHs from saturated aerobic granules. This suggested that, when only real slop was fed to R1, the main mechanism of hydrocarbon removal was the bio-adsorption. In R2, although it was observed a higher concentration of bio-adsorbed hydrocarbons during Phase V^b compared to the previous phase (Fig. 6j), the decreasing trend of TPHs concentration both in liquid phase and adsorbed into the granules, discussed by analysing the data in Fig. 5b and d, denoted a biological adaptation of microorganisms to recalcitrant hydrocarbons. These results corroborated the theory for which to obtain aerobic granules directly with industrial wastewater is preferable than to inoculate a system with preformed mature granules grown with other substrates. In this way, it is possible to enhance the adaptation and the specialization of proper microorganisms able to metabolize the recalcitrant substrates.

3.5. Applications of AGS for the treatment of slop wastewater

Among the technology-oriented applications of this work, it is possible to mention two main categories: (1) applications in-situ on-board of the oil tankers, thanks to the compactness of the AGS systems and the operational flexibility of SBR reactors; (2) applications ex-situ on-site or off-site. Regarding the on-site applications, it possible to include industrial wastewater treatments inside the refineries areas. On the other hand, off-site treatments could be operated in proper wastewater treatment plants out of the refineries. In this case, the transportation of slop wastewater from the place of production to the treatment area could constitute an environmental impact. Therefore, it is preferable to treat slop wastewater directly on-board or in the wastewater treatment plant of refineries. From an economic point of view, although there are various low-cost biomaterials to be used as adsorbents in place of commercial activated carbon (Table S1), aerobic granular sludge does not act as a bio-adsorbent only. Since aerobic granules are made of microorganisms, in addition to a first step of bio-adsorption of recalcitrant contaminants, a second step of biodegradation of the adsorbed fraction may occur, as demonstrated in this study. Therefore, since aerobic granules form spontaneously and continuously inside the reactor and since they are able to biodegrade recalcitrant compounds, such as hydrocarbons as in R2 in this work, the bio-adsorbent sites can be regenerated continuously. This could represent a remarkable advantage of AGS technology respect to commercial activated carbon as adsorbent.

4. Conclusions

This work compared two strategies for hydrocarbons removal from slop wastewater: (i) application of mature salt-adapted AGS (R1); (ii) direct cultivation of AGS with industrial slop wastewater (R2). Although in both the systems AGS showed good physical properties, the direct cultivation with slop highlighted better results in terms of TPHs removal, as occurred in R2. During granulation, the higher EPS production enhanced both the bio-aggregation and the bio-adsorption of hydrocarbons, thus increasing the contact time with the microorganisms. This dual mechanism implied both a physical and a biological selection, thus favouring the development of robust and mature AGS where microorganisms were able to biodegrade TPH.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2019.122083.

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