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Perspectives of intracellular polymers in functional evaluation of the microbes for EBPR

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ABSTRACT

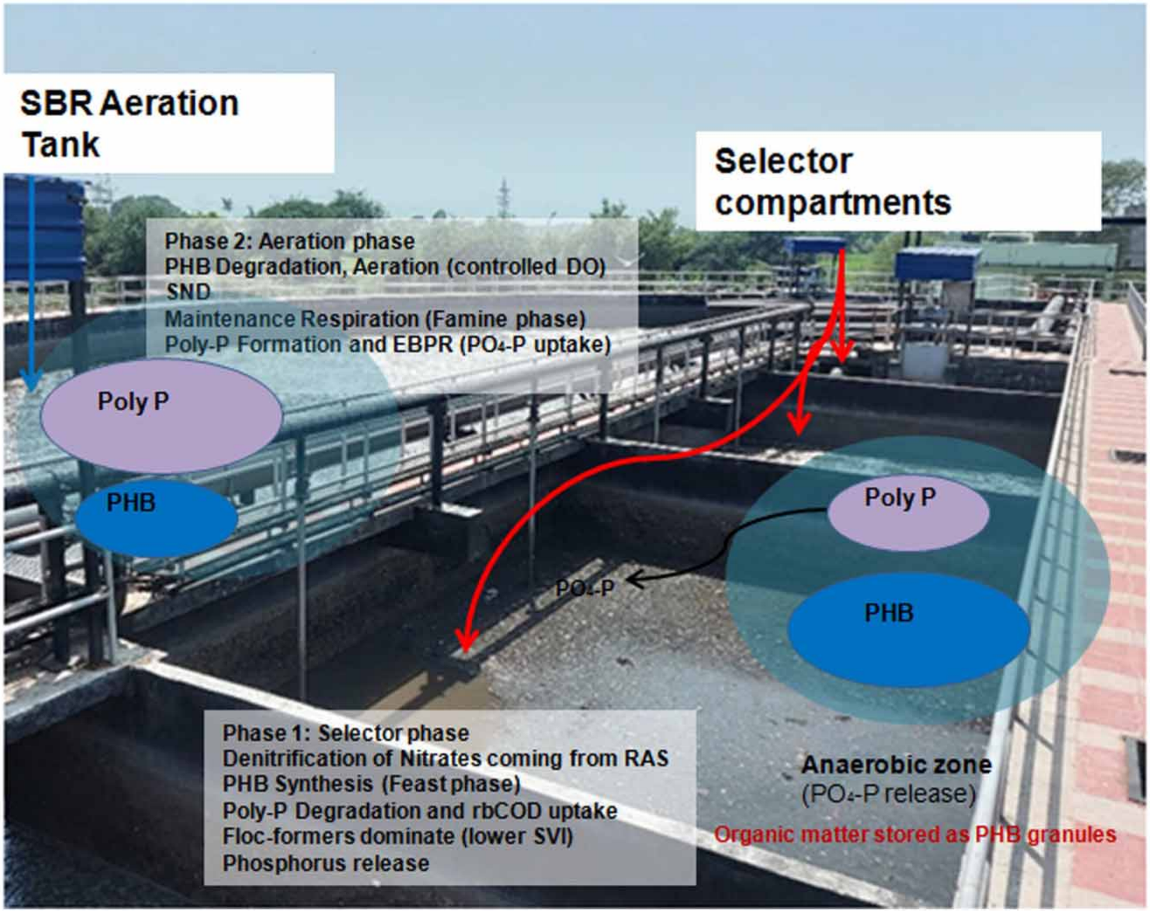
To substantiate and interpret the performance of the Enhanced Biological Phosphorus Removal (EBPR) processes with simultaneous nitrogen removal in five full-scale sequencing batch reactors (SBR) systems (with or without pre-anoxic/anaerobic selector) across India, conventional microscopic examinations were performed. Regular examining and cyclic behavior evaluation studies specified that these systems worked for EBPR with effectiveness depending on the wastewater quality and operational steadiness. Treatment with Neisser stain for identifying polyphosphates (poly-P) and Sudan black B stain for observing poly-β-hydroxybutyrates (PHB) granules showed that the enriched biomass of the SBR plants was very diverse concerning morphology, residing populations of traditional rod-shaped PAOs, tetrad (or Sarcina-like cells) forming organisms (submitted as TFOs instead of GAOs), diplococci-shaped cells, and staphylococci-like clustered populations (CC), including few filaments which correlate well with biochemical processes undergoing in SBR plants. SBR plants with readily biodegradable chemical oxygen demand (rbCOD) fraction in COD $>$ 16% and rbCOD/TP ~10–20 in Varanasi, Mumbai, and Gurgaon, respectively, have performed for $>$20% EBPR (~77.8%, ~76.6%, and ~84.8% TP removal, respectively) as well as $>$85% Simultaneous Nitrification and Denitrification (SND). This study can open novel dimensions for optimization by relating microscopic observations (qualitative examination) with the processes undergoing in the plants under varied physicochemical parameters.

Key words: coccoid clusters, denitrification, dissolved oxygen, enhanced biological phosphorus removal, glycogen accumulating organisms

HIGHLIGHTS

- Five full-scale SBR plants are analyzed for the study.
- STPs at Varanasi, Mumbai, and Gurgaon achieved $>$75% TP removal.
- Opens novel dimensions for optimization relating to microscopic studies and biochemical processes.
- Strictly upholding anaerobic conditions in the anaerobic zone enhances EBPR.
- The biomass of the SBR plants was very diverse.

GRAPHICAL ABSTRACT



INTRODUCTION

There have been a variety of biological treatment plants across India, mainly consisting of SBRs, which perform excellently in achieving simultaneous nutrient (N and P) removal, organic matter (COD and BOD), and total suspended solids (TSS) removal. But besides nitrogen removal, phosphorus removal requires a balanced connection between controlled operating conditions and prevalence of polyphosphate accumulating organisms (PAO) against glycogen accumulating species/organisms (GAO), which constitutively results in enhanced biological phosphorus removal (EBPR) (Gebremariam *et al.* 2011). According to the initial reports of the EBPR phenomena, many different full-scale plant configurations have been proposed and constructed. They have developed practically at varying operating conditions but the knowledge of fundamental conceptions of the microbiology involved based on conventional microscopic techniques is still less/limited, as the structure of the EBPR is complicated (Mino *et al.* 1998; Dulekgurgen *et al.* 2011). In the lack of this vital information, it is possible that these plants may not work under the most favorable conditions, specifically for EBPR (Seviour *et al.* 2003; Gebremariam *et al.* 2011). The N and P concentrations in the influent differ with its source and the cultural manners of particular societies. Therefore, it appears sensible to summarize our present state of perceptive of EBPR microbiology and consider what still requires to be known if we propose and run enhanced EBPR systems along with Nitrogen removal in the future.

Many researchers suggested that the following operational conditions are obligatory for successful EBPR (Seviour *et al.* 2003):

1. Anaerobic zone which receives the initial influent including the readily degradable carbon and energy sources,
2. It is a necessity to limit the nitrate concentration coming in this anaerobic zone by incorporating an anoxic zone in the design because, in its occurrence, EBPR can be unsuccessful as the denitrifying bacteria were

believed able to anaerobically respire and diminish the availability of organic substrates, making them no longer accessible for the PAOs,

3. Strictly upholding anaerobic conditions in the anaerobic zone,
4. The recycling of the biomass through alternating anaerobic: aerobic conditions.

The significant aspects affecting EBPR's performance include organic loading rate, total hydraulic retention times (HRT), anaerobic HRT, solids retention times (SRT), dissolved oxygen (DO), oxidation-reduction potential (ORP), and influent characteristics such as COD, solids content, and C/N or C/P ratio (Metcalf and Eddy Inc 2003; Gautam *et al.* 2016). By controlling these constraints, the SBR can be constructed for functions such as organic carbon oxidation, suspended solids removal, nitrification, and denitrification, and phosphorus removal simultaneously (Metcalf and Eddy, Inc. 2003). However, the standard agreement is that in the anaerobic phase, some bacteria very quickly assimilate short-chain volatile fatty acids (VFA) developed by the action of chemoorganotrophic fermentative bacteria (Fuhs & Chen 1975; Wentzel *et al.* 1985). These are stored as poly-β-hydroxy alkanoates (PHA), whose chemical composition depends on the type of organic substrate assimilated. Hence with acetate assimilation, poly-β-hydroxybutyrate (PHB) is synthesized and increases in parallel. Synthesis of PHA requires a source of reducing power; that is, glycogen, an intracellular storage compound synthesized aerobically by the PAO, was catabolized anaerobically to produce electrons for PHA creation according to the Mino model. Glycogen has a crucial role in EBPR as it adjusts the redox balance of the PAO, which may be indispensable for allowing the anaerobic assimilation and metabolism of readily biodegradable substrates in full-scale systems (Mino *et al.* 1998; Seviour *et al.* 2003; Sathasivan 2009). In the EBPR process, intracellular poly-P content drops. An increment in phosphate levels in the bulk liquid can be identified following its release from the biomass in the anaerobic stage (Bunce *et al.* 2018). Then in the next aerobic phase, PHA levels in the biomass fall simultaneously with a decrement in phosphate levels in the bulk liquid and growth in biomass P levels as poly-P, which can be >15% of cell dry weight (Mino *et al.* 1998; Seviour *et al.* 2003). If a biomass sample taken from the aerobic reactor is examined microscopically, cells staining for poly-P are abundant, but no/lesser intracellular PHA can be detected by staining. On the other hand, cells in biomass from the anaerobic reactors stain for PHA but not poly-P. In addition, the cells appearing as large coccobacilli in pairs representing staining responses are usually arranged into significant floc-combined clusters or may sometimes be loosely scattered all over the biomass (Bond *et al.* 1999; Seviour *et al.* 2003). It is supposed that these chemical transformations are employed only by the PAO arranged in this typical way and believed that excellent EBPR performance is frequently related to the emergence of big dense clusters in the biomass (Seviour *et al.* 2003). Poly-P granules stain pink with methylene blue and so are referred to as metachromatic. Few studies have been published on its association and intracellular sites in correlation to EBPR sludge (Kornberg *et al.* 1999). The presence of nitrate is thought to provide denitrifying bacteria with an opportunity to remove the PAO's selective advantage in these systems by out-competing them for the metabolizable substrates available there (Barker & Dold 1996). Nitric oxide also prevents anaerobic P release in EBPR sludge by hindering the adenylate kinase production involved in poly-P degradation (Van Niel *et al.* 1998). However, it has often been exhibited that in the deficiency of any exogenous carbon sources in the anaerobic zone, EBPR may take place in the presence of nitrate using intracellular storage compounds like PHA as carbon and energy sources to incorporate P and generate poly-P, as PAOs, but with nitrate not oxygen as their terminal electron acceptor in the process known as the recovery of intracellular glycogen (Seviour *et al.* 2003; Bunce *et al.* 2018). Some evidence suggests that nitrite may be used by denitrifying polyphosphate accumulating organisms (DNPAO) in P uptake (Meinhold *et al.* 1999; Serafim *et al.* 2002). The attractions of utilizing such populations in wastewater treatment comprise the possibility of achieving simultaneous removal of N and P, producing reduced sludge, and with no requirement for aeration, having a process that is less costly to operate.

EBPR prevails if PAOs dominate over GAOs. A big competition occurs between PAOs and GAOs, and EBPR failure occurs due to the prevalence of the 'G-Bacteria' or GAOs as they cannot accumulate poly-P during the aerobic phase (Mielczarek 2012). This term describes a morphotype of cocci, which often appears in activated sludge/SBRs and is now observed as very diverse phylogenetically. Cells are distinctively arranged in tetrads or clusters and referred to as tetrad-forming organisms (TFO) or Sarcina-like cells with a diameter of 1.5–3 μm (Tsai & Liu 2002; Dulekgurgen *et al.* 2011). Regardless of the system, it can be seen running with excessive COD (Dulekgurgen *et al.* 2003). Several bacteria that emerged as large cocci in their biomass samples were regarded as responsible for these chemical changes because of their supremacy. It was observed that these clusters were highly dense, and it was impossible to decide whether they were groups of separate cocci or collections of tetrads/sarcina-resembling organisms. These cells were expressed not in terms of a morphotype, however as a phenotype, the supposed glycogen-accumulating organism (GAO) (Seviour *et al.* 2003; Shi 2011). However, it was also interpreted that as the coccoid cells in these coccoid clusters did not store acetate at the anaerobic period, they were supposed not to be GAOs, but instead were guessed to be possible ordinary facultative heterotrophs (OHOs), denitrifiers, or even nitrifiers (Dulekgurgen *et al.* 2003, 2011; Dülekgürgen & Artan 2006).

Most of the full-scale studies focused on absolute microbiology of the EBPR sludge, though lack of unambiguously identified PAO isolates confines the studies to understanding the enzymology and phylogeny of this functional group of bacteria, which will help elucidate the biochemical characteristics of these microorganisms (Dulekgurgen *et al.* 2003, 2011). The presence of non-poly-P bacteria also complicates the research on EBPR performance of the PAOs, the relationship between the PAOs and the others, and their effect on overall treatment performance (Crocetti *et al.* 2000). Even the basics of the EBPR process in lab-scale and pilot-scale studies remain incomprehensible to date (Dulekgurgen *et al.* 2003, 2011). Moreover, there are limited pilot-scale/full-scale SBR-based studies, which closely meet the N and P removal simultaneously (Srivastava *et al.* 2021). Therefore, knowledge of peculiar diversity related to EBPR/or EBPR with N removal demands additional perspectives from conventional techniques; that is, differential staining and floc morphological studies based on fundamental microbiological and biochemical aspects of the EBPR phenomenon that mark a significant contribution in the understanding concerning these processes and mechanisms.

This study helps in making clear descriptions of microbiology involved with biochemical processes in different pre-anoxic selector-based (Figure 1) or no selector-based full-scale plant configurations focused on biological N and P removal simultaneously. The objectives of the research are to find a relationship among biological nutrient (N and P) removal via SND and EBPR processes, respectively; morphological and phenotypic characteristics of biomass; and presence of functional organisms (analyzed through qualitative approaches, i.e., chemical-staining and conventional light-microscopy) in five full-scale SBR plants. The aim of the study also includes determining particular operational (biochemical and physicochemical) parameters/conditions for practicing enhanced biological phosphorus removal along with the biological nitrogen removal based on the experiences from full-scale SBR plants of India. The particular overall methodology of this study is shown in Figure 2.

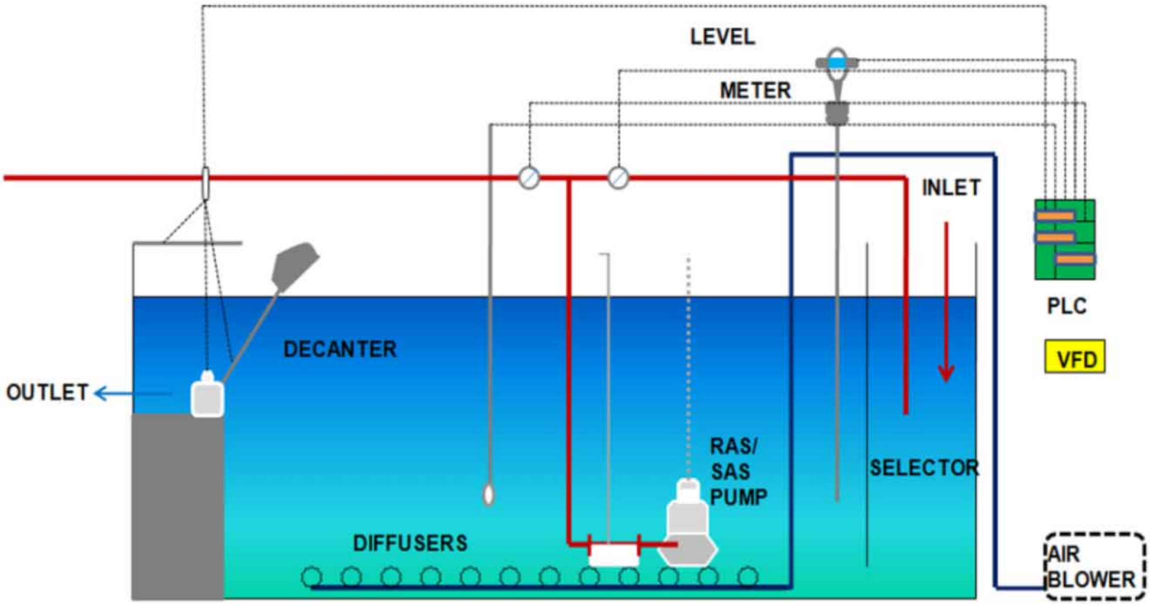


Figure 1 | A typical representation of selector-attached SBRs.

The studies on EBPR with biological nitrogen removal remain less (Meyer *et al.* 2005), and evaluating such systems in microbiological and phenotypic traits is even further rare (Dulekgurgen *et al.* 2003). Applications of the advanced molecular techniques for microbial communities' identification in EBPR systems has been well acknowledged, but the categorization of microorganisms as PAOs or GAOs as functional groups, rather than

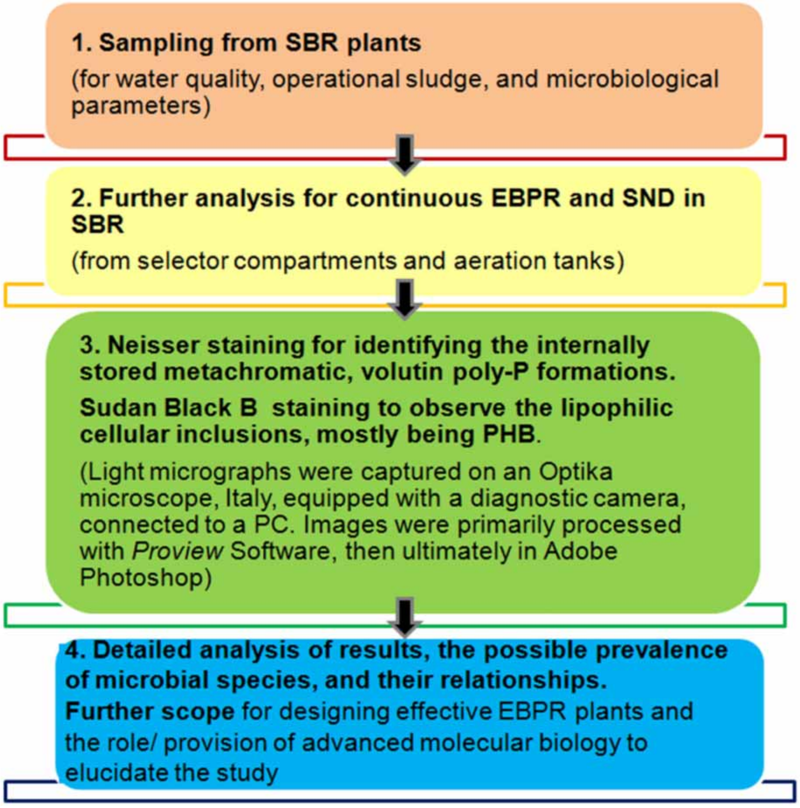


Figure 2 | Flow chart presenting the methodology of the study.

phylogenetic, and the co-existence of these two functional groups in EBPR systems, obscure evaluation of the systems (Dulekgurgen *et al.* 2011). The qualitative analysis needs to be supported by biochemical performance evaluation as well as by morphological and phenotypic descriptions to accurately identify the populations of the microflora contributing to the observed transformational processes. It also determines the extent of involvement of various populations and can be realized by performing a combined set of studies (Dulekgurgen *et al.* 2011). Therefore, the current study probes over three novel dimensions of full-scale SBRs, which can be beneficial for further researches:

1. Based on the microscopic observations, several microbial species were recognized; that is, TFOs and CCs (having characteristics of GAOs), PAOs, and OHOs, as well as intracellular polymers like PHBs and polyphosphate globules in full-scale SBRs. These kinds of comprehensive phylogenic studies along with quantitative analysis and biochemical performance evaluation are lacking in full-scale EBPR plants.
2. Differences in the level of performance between selector-based and non-selector-based full-scale SBRs concerning biological nutrient (N and P) removal, sludge morphology, flocculation, and microbiological aspects were studied.
3. For both the nutrients; that is, N and P, removal efficiencies were evaluated and analyzed on microbiological perspectives based on SND and EBPR processes. Generally, these kinds of comprehensive studies are lacking in developing countries for nitrogen-removing EBPR plants.

MATERIALS AND METHODS

Samples from the influent and effluent by the end of two subsequent cycles and from mixed liquor at the end of filling and anaerobic phases were collected, filtered, and preserved (when necessary) for analyses. The biochemical performance of the SBR was monitored by daily measurements of sludge volume index (SVI), total suspended solids (TSS), volatile suspended solids (VSS), ammonia-N (NH_4-N), nitrate-N and nitrite-N (NOx-N), total nitrogen (TN), chemical oxygen demand (COD), biochemical oxygen demand (BOD), total phosphorus (TP) and orthophosphate (PO_4-P). These analyses were carried out according to Standard Methods of APHA (APHA 2005). The readily biodegradable chemical oxygen demand (rbCOD) fraction is determined by the flocculation-filtration method (Wentzel *et al.* 2000). In addition, the total P content of the biomass was determined in these aerobic mixed liquor samples via performing sulfuric acid-nitric acid digestion followed by the stannous chloride method. EBPR was calculated as TP removed more than ~2.7% of PO_4-P uptake as VSS in the sludge. To identify the biomass's macro-structure and determine the essential morphological diversity, bright-field microscopy was performed. Light micrographs were captured on an Optika microscope (Italy), equipped with a diagnostic camera, connected to a PC. Images were primarily processed with 'PROVIEW' Software, then ultimately in Adobe Photoshop. For that purpose, Poly-P and PHB staining procedures were performed on samples collected at the different periods within cycles. Although not specific for poly-P, but has been widely used for that purpose, Neisser's staining (Methylene Blue and Crystal Violet for staining poly-P globules, and Bismark Brown or Chrysoidin Y for counter-staining) was used for the identification of the internally stored metachromatic, volutin poly-P formations. To understand and observe the lipophilic cellular inclusions, mostly being PHB, samples were stained with Sudan Black B and then with Safranin O for counter-staining (Dulekgurgen *et al.* 2003; Srivastava *et al.* 2021). All the staining procedures were carried out according to the manual (USEPA 1987; Jenkins *et al.* 1993; Dulekgurgen *et al.* 2011).

The four full-scale SBR plants were treating municipal wastewater with capacities of 3 million liters per day (MLD) (Roorkee), 14 MLD (Prayagraj), 120 MLD (Varanasi), and 87.5 MLD (Mumbai) and have a configuration as pre-anoxic multi-cell selector attached SBRs (Figure 1) comprising C-Tech-based automated technologies with proper tertiary treatment facilities (Figure 1S, supplementary information). The fifth full-scale SBR, with a capacity of 50 MLD, was located in Gurgaon, Haryana, and treating municipal and industrial wastewater both in a mixed ratio of 2:3, respectively. The SBR was designed based on Aquatech SBR treatment facility having five zones in a four hour cycle; that is, mix-fill (30 minutes); react-fill (30 minutes); react/aeration (90 minutes); settle (45 minutes) and decant (45 minutes). The location and capacity of all the treatment plants are shown in Table 1S (Supplementary Information).

RESULTS AND DISCUSSION

The results in this study have been interpreted in two forms: performance evaluation analysis based on biochemical parameters. The other is microbial examination/analysis through light microscopic studies, which brings an outcome to the survey emphasizing the comprehensive correlation of/between the observations.

Performance evaluation of SBR plants

The 120 MLD SBR, 87.5 MLD SBR, and 50 MLD SBR plants at Varanasi, Mumbai, and Gurgaon, respectively, have been working excellently in accomplishing EBPR (>20%). However, suppose all the parameters fit effectively, including rbCOD/TP or COD/TP ratios, anaerobic conditions (optimized DO and ORP levels for possible fermentation of rbCOD into VFAs), least recycle of nitrates, and lesser recycle ratios in the anaerobic zones/pre-anoxic selector compartments and the possibility of survival of PAOs other than GAOs. In that case, it supports the phenomena of EBPR. A phenomenal example of it can be noticed in Mumbai and Varanasi SBR plants, which showed higher TP removal and TN removal than other selector-based or non-selector-based SBRs (Table 1).

Identification of tetrads/sarcina kind of cells, coccoids, OHOs, and rod-shaped possible PAO structures through qualitative means, i.e., Neisser and Sudan Black B staining

There may be four groups of cells that can be represented as TFO, type 2 (TFO2), TFO type 1 (TFO1), rod-shaped Bacilli, and coccoid clusters, which were observed after staining. A few diplo-coccoid cells were also detected but rare (Mino *et al.* 1998; Lindrea & Seviour 2002; Tsai & Liu 2002; Dulekgurgen *et al.* 2003).

The samples from the Roorkee SBR plant signify a possible diversity of PAOs, TFOs, CC, OHOs, nitrifiers, and denitrifiers as described in Table 2. Analysis of biochemical parameters interprets that COD/TP and COD/TN ratios were found as 65.6 and 11.8 respectively, contributing to >40% TP and >71% TN removal efficiencies in the plant. The COD and BOD removal was >95%, sCOD and sBOD reduction was >92%. Also, in the anoxic/anaerobic phase (selector) ~82% of sCOD removal (25 mg/L in the selector's compartment) was observed, which proposed a clear C-storage mechanism operating under anaerobic states; a feature representative both for the PAO- and the GAO phenotypes (Dulekgurgen *et al.* 2011). Ammonia removal was 96.8%, which showed a diverse possibility of nitrifiers, denitrifiers, and OHOs contributing to 76.5% SND efficiency of the plant. The rbCOD/TP is known to affect the prevalence of PAOs, which should be <10–20 for better EBPR as it can be tested by calculating from Table 1 (Broughton *et al.* 2008). Images from the sludge samples of SBR in Roorkee, Uttarakhand, India (3 MLD capacity) are shown in Figure 3.

Table 1 | (A) Influent design parameters and (B) average temporal variations in water quality parameters (biochemical processes) and operational parameters in SBR plants used in the study

S. No.	Plants capacity and location	3 MLD SBR Roorkee, Uttarakhand	14 MLD SBR Salori, Prayagraj, UP	120 MLD SBR Varanasi, UP	87.5 MLD SBR Mumbai, Maharashtra	50 MLD SBR Gurgaon, Haryana
A. Influent design parameters						
1	Type of wastewater (municipal/industrial/mixed)	Municipal	Municipal	Municipal	Municipal	Mixed
2	Technology	Cyclic activated sludge (C-Tech)	Cyclic activated sludge (C-Tech)	Cyclic activated sludge (C-Tech)	Cyclic activated sludge (C-Tech)	Aqua-Tech SBR
3	Average actual flow rate (m^3/d)	1,880	14,000	22,296	36,624	2,083
4	Aeration tank volume in m^3 (no. of basins)	2,414.5 (2)	9,210.5 (2)	7,1911 (6)	5,2424.4 (6)	~39,600 (4)
5	With or without anoxic selector	With selector	With selector	With selector	With selector	Without selector
6	Volume in m^3 (no. of compartments in selector (if present))	168.9 (3)	1,579.5 (3)	1,549.8 (9)	1,234 (1)	–
7	Total HRT (h) and anaerobic HRT (h)	18.1 and 1.0	15.8 and 2.71	14 and 0.75	14.4 and 0.75	~ 19 h and 0.5–0.75
8	SRT (d)	15.2	20	10.4	10–20	10–20
9	Cycle time (h)	4	4	3	3	4
10	Recycle ratio (%)	25%	40% (can vary)	18%	15–20%	Not applicable

		3 MLD SBR Roorkee, Uttarakhand		14 MLD SBR Salori, Prayagraj, UP		120 MLD SBR Varanasi, UP		87.5 MLD SBR Mumbai, Maharashtra		50 MLD SBR Gurgaon, Haryana	
		Inlet	**Outlet**	**Inlet**	**Outlet**	**Inlet**	**Outlet**	**Inlet**	**Outlet**	**Inlet**	**Outlet**
B. Average temporal variations in water quality and operational parameters in the influent and effluent											
1	pH	7.2	7.4	7.6	7.9	7.0	7.4	6.8	7.2	7.0	7.2
2	Temperature (average/or during sampling)	25.4	24.9	26.2	26.1	22.0	22.0	25.0	24.9	26.2	26.2
3	COD	400	18	273	8	326	18	290	35	612	39
4	sCOD	140	10	138	5	107	7	105	12	152	10
5	BOD	163	6	147	4	230	6	127	10	363	10
6	sBOD	63	3	59	2	56	3	46	4	90	7
7	rbCOD	46	–	22	–	55		92	–	110	–
8	TN	34	9.7	33.8	11.8	58	10.0	30	10.5	58.3	28.9
9	NH_4-N	22	0.7	18.9	1.6	43.7	1.6	24.5	5.8	39.8	20.0

10	NO_3-N	1	6	0	9	1.1	3.7	1	3	1.9	2.2
11	TP	6.1	3.6	3.6	1.9	4.5	1.0	6.4	1.5	5.9	0.9
12	PO_4-P	2.8	1.8	1.7	1.3	3.3	0.6	3.5	1.0	3.6	0.3
13	TSS	237	9.4	132	5	312	8	162	20	313	11
14	VSS	128	5.0	68	2	172	4.6	81	8	250	6
15	Alkalinity	350	260	520	480	480	320	270	180	520	480
17	Average enhanced uptake (%) excluding the uptake by BOD_5	14.3%		6.4%		26.7%		56.7%		23.2%	
18	Average MLSS in SBR aeration tank (mg/L) and SVI (mL/g)	7,189 and 48		3,800 and 79		3,778 and 34		2,170 and 83		3,260 and 100	
19	TN% in sludge	1.6–2.7		1.5–2.2		1.6–3.5		3.6–4.6		1–3	
20	TP% in sludge	2.5–3.5		2–3		4–6		5–7		4–5	
21	DO in aeration tank and selector (/extreme anaerobic phase) (mg/L)	1.5 ± 0.9 and 0.14 ± 0.1		2.0 ± 0.6 and 0.2 ± 0.1		1.4 ± 1.2 and 0.12 ± 0.10		1.6 ± 0.8 and 0.1 ± 0.09		1.6 ± 0.4 and 0.2 ± 0.1	
22	ORP in aeration tanks and selector (/extreme anaerobic phase) (mV)	105 ± 45 and -90 ± -24		99 ± 50 and -70 ± -40		$+130 \pm 70$ and -208 ± -90		200 ± 90 and -220 ± -50		150 ± 35 and -150 ± -50	

Table 2 | Brief description and interpretation of micrographs shown in Figure 3

Figure 3	Morphology	End of anaerobic period	End of aerobic period	Observation	Abbreviation
Panels (A to E)	Rod-shaped cells	P (−−−) PHB (++−)	P (++ − PHB (+−−)	Rod-shaped cells are Neisser positive and PHB positive (anaerobic condition: PHB more; poly-P less and aerobic condition: PHB less; poly-P more)- possibly PAOs (P)	PAO
Panels (I and J)	Tetrads/ Sarcina-like cells	eP (+−−) PHB (+−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative- possibly GAOs (eP). It is possible that GAOs detected as poly-P (+) in the form of tetrads can be proficient in performing EBPR at meager rates.	TFO1
Panels (C, F, G, H, I)	Coccoid-clusters	eP (++−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – may be GAOs or possible ordinary facultative heterotrophs (OHOs), denitrifiers, even nitrifiers (eP)	CC

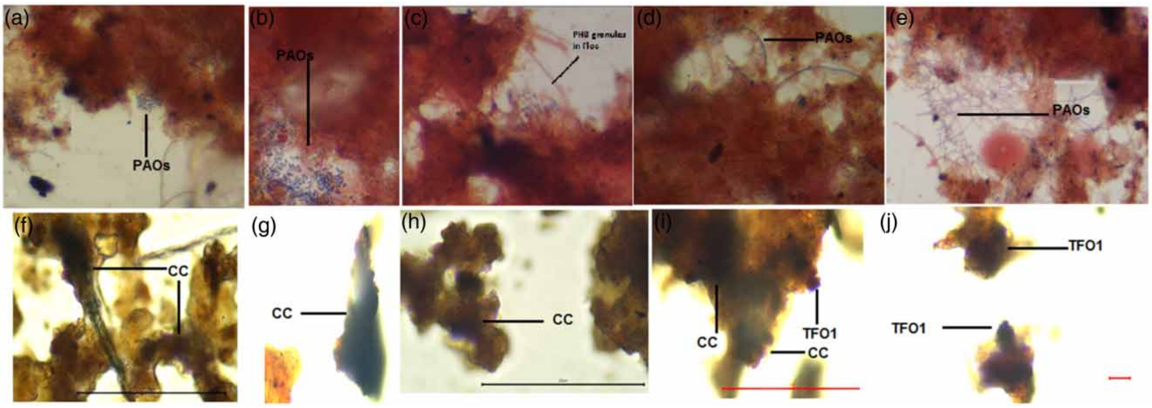


Figure 3 | Bright-field micrographs of samples from Roorkee, SBR (with selector) biomass: Case I. Panels [a] to [e]: end of the anaerobic period and panels [f] to [j]: end of the aerobic period. Samples in panels [f] to [j] were treated with Neisser-stain for poly-P visualization and samples in panels [a] to [e] were treated with Sudan Black B to stain PHB inclusions. Elucidation of these micrographs is summarized in Table 2. Panels [a] to [e] are showing PHB granules. Panels [d] and [e] show PHB granules in the filaments. Black-colored poly-P stains appear due to either longer exposure time of crystal violet and methylene blue solution or due to a higher % of TP (as poly-P) in sludge biomass.

Similarly, the images from the sludge samples of SBR in Salori, Prayagraj (Allahabad), UP, India (14 MLD capacity) have been shown in Figure 4. The first four images are Sudan Black B stained micrographs for PHB identification, and the other nine images are Neisser stained micrographs for detecting poly-P inclusions. Samples from Salori, Prayagraj (UP) SBR plant demonstrated a mixed composition of PAOs, CC, and TFOs with nitrifiers and denitrifiers (Table 3). The rbCOD/TP ratio was 6.1 and COD/TP of 75.8 provided 47.2% TP removal and COD/TN ratio of ~8.1 contributed to 65.1% TN removal and 91.5% ammonia removal.

The sludge samples from the Varanasi SBR plant showed significantly diverse PAOs, nitrifiers, and denitrifiers with fewer structures identified as TFOs, and CC (Table 4). Excellent TN and TP removal of 82.8% and 77.8% were observed at COD/TN and COD/TP ratios of 5.6 and 72.4, respectively. The rbCOD/TP ratio of 12.2, <-200 mV ORP in the anaerobic compartments of the selector and controlled DO and OUR operations altogether contributed to higher SND efficiency (93.8%) and EBPR (26.7%) in the SBR plant. The microscopic pictures from the sludge samples of SBR in Goithaha, Varanasi, UP, India (120 MLD capacity) after staining are shown in Figure 5.

The images from the sludge samples of the SBR in Koparkhairane, Mumbai, Maharashtra, India (87.5 MLD capacity) have been shown in Figure 6. The first six images are Sudan Black B stained micrographs for PHB identification, and the other six images are Neisser stained micrographs for detecting poly-P inclusions. Sludge samples from the Mumbai SBR plant also showed various microbial populations belonging to possibly PAOs,

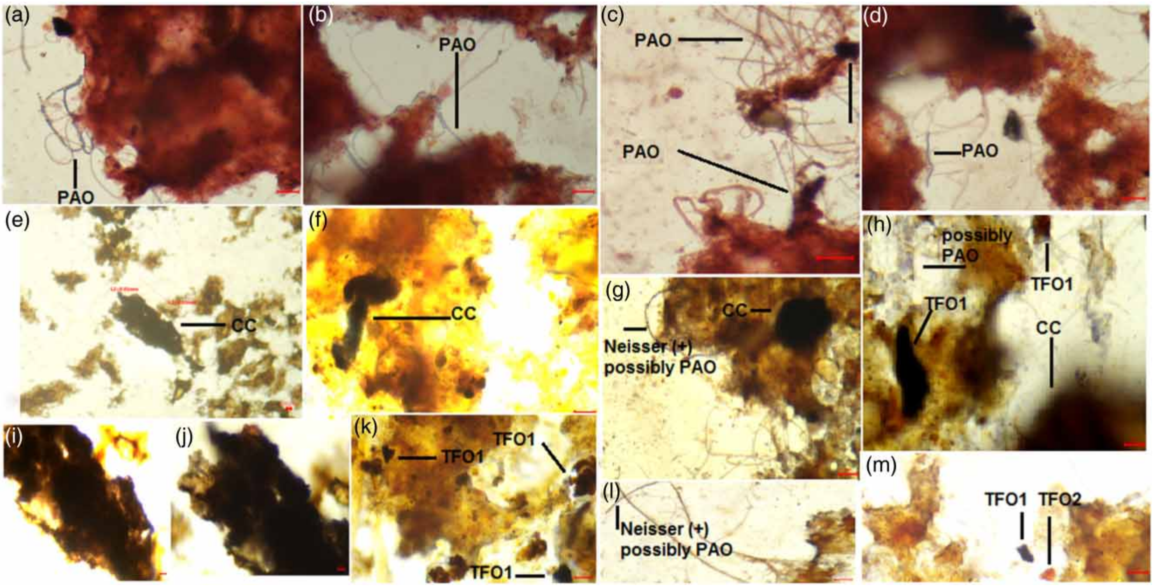


Figure 4 | Bright-field micrographs of samples taken from Salori SBR (with selector) sludge: the panels [a], [b], [c], and [d] were obtained at the end of the anaerobic period and show PHB granules in the filaments, and the panels [e] to [m] were obtained at the end of the aerobic period. Samples in panels [e] to [m] were examined with Neisser-stain for poly-P images and samples in panels [a] to [d] were treated with Sudan Black B to stain PHB inclusions. The description of these micrographs is summarized in Table 3. The panels [I], [J] are the magnified images of panel [E] at 100X to visualize the CC more in depth. Black-colored stains appear due to either longer exposure time of crystal violet and methylene blue solution or higher % of TP (as poly-P) in sludge biomass.

Table 3 | Brief description and elucidation of micrographs shown in Figure 4

Figure 4	Morphology	End of anaerobic period	End of aerobic period	Observation	Abbreviation
Panels (A to D, G, H, L)	Rod-shaped cells	P (−−−) PHB (++−)	P (++−) PHB (+−−)	Rod-shaped cells are Neisser positive and PHB positive (anaerobic condition: PHB more; poly-P less and aerobic condition: PHB less; poly-P more) – possibly PAOs (P)	PAO
Panels (H, K, M)	Tetrads/ Sarcina-like cells	eP (+−−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative- possibly GAOs (eP)	TFO1
Panels (M)	Tetrads/ Sarcina-like cells	eP (−−−) PHB (+−−) Gram (−−−)	eP (−−−) PHB (−−−) Gram (−−−)	Do not stain at all, gram- negative – possibly GAOs (eP)	TFO2
Panels (E, F, G, H, I, J)	Coccoid-clusters	eP (++−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – can be GAOs, ordinary facultative heterotrophs (OHOs), Denitrifiers, and Nitrifiers (eP)	CC

OHOs, nitrifiers, and denitrifiers with fewer structures identified as TFOs, and CC (Table 5). This plant showed optimal COD/TN (9.7) and COD/TP (45.3) ranges and resulted in TN and TP removal of 65.0% and 76.6%, respectively. The rbCOD/TP ratio of 14.4, <-220 mV ORP in the anaerobic compartments of a selector, longer sewer lines to produce sufficient VFAs, and controlled DO and OUR operations altogether contributed to higher EBPR (56.7%) along with excellent SND efficiency (89.3%) and in the SBR plant. The organic matter removal was >87%, and sCOD and sBOD removal % were 88.6 and 91.3, respectively.

The sludge samples from Gurgaon (Haryana) SBR plant also revealed a possibility of mixed composition of PAOs, CC, and TFOs with nitrifier and denitrifier diversity. The rbCOD/TP ratio was 18.6 and COD/TP of

Table 4 | Brief description of micrographs shown in Figure 5

Figure 5	Morphology	End of anaerobic period	End of aerobic period	Observation	Abbreviation
Panels (A, B, C, D, E, F)	Rod-shaped cells	P (−−−) PHB (++−)	P (++−) PHB (+−−)	Rod-shaped cells are Neisser positive and PHB positive (anaerobic condition: PHB more; poly-P less and aerobic condition: PHB less; poly-P more) – possibly PAOs (P)	PAO
Panels (E, H)	Tetrads/ Sarcina-like cells	eP (+−−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – possibly GAOs (eP)	TFO1
Panels (E, G, H)	Coccoid-clusters	eP (++−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – GAOs, possible ordinary facultative heterotrophs (OHOs), denitrifiers, and nitrifiers (eP)	CC

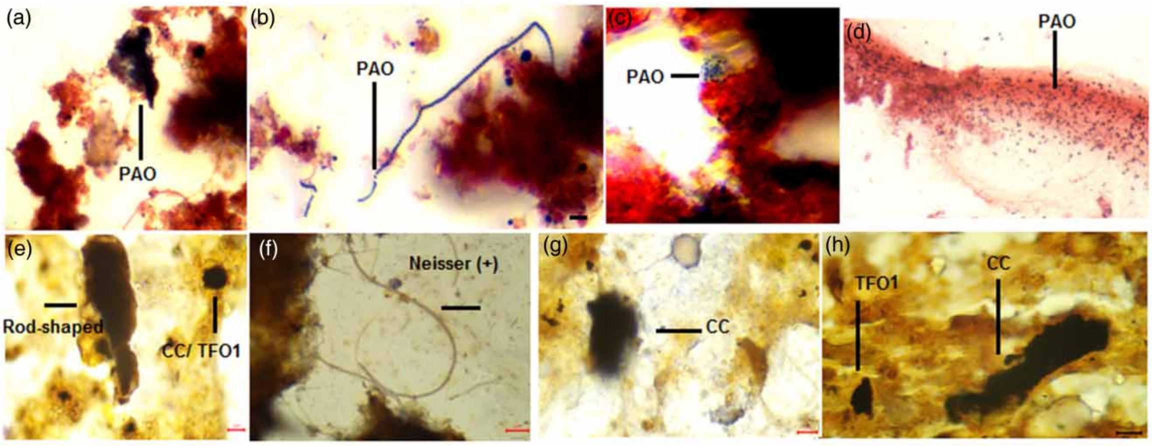


Figure 5 | Bright-field micrographs of samples from Varanasi SBR (with selector) biomass: Case I. Panels [a], [b], [c], and [d]; end of the anaerobic period. Panels [e], [f], [g] and [h]; end of the aerobic period. Samples in panels [e], [f], [g], and [h] were treated with Neisser-stain for poly-P visualization, and samples in panels [a], [b], [c], and [d] were treated with Sudan Black B to stain PHB inclusions. Conclusive analysis of these micrographs is summarized in Table 4.

103.6 provided 84.7% TP removal and 23.2% EBPR. However, it can be possible that the biodiversity of nitrifiers was quite lesser in comparison to other microbes, and the COD/TN ratio of ~10.5 contributed to only 50.4% TN removal and 49.7% ammonia removal in the plant. Microscopic images from the sludge samples of SBR in Gurgaon, Haryana, India (50 MLD capacity) have been shown in Figure 7 and their interpretation is provided in Table 6.

By combining the results of biochemical analyses from the different WWTPs (SBRs) described in Table 1 and microscopic observations from bright-field micrographs, several points were interpreted:

1. For the Varanasi SBR plant, the micrographs (panels A to F) confirmed appearances of PAOs (strong-stained positive PHBs are visible at the end of the anaerobic phase), and panels E, G, and H showed some possible structures of TFO1 (possibly GAOs) and CCs (may be the occurrence of diverse communities like OHOs, GAOs, nitrifiers, and denitrifiers). The descriptions validated higher biological nutrient removal efficiencies in the plant (TN and TP removal of 82.8% and 77.8%, respectively). Several design and influent wastewater parameters include rbCOD/TP of 12.2, rbCOD/COD (17%), DO (0–2.5 mg/L), ORP in the extreme anaerobic zone (<-200 mV) and aerobic tank (>100 mV), anaerobic HRT (0.75 h) and lower nitrates recycling rates (18%) also substantiate for high average EBPR (>26%) as well as SND (>90%) in the plant (Srivastava *et al.* 2021). EBPR biomass justified with advanced settling properties and denseness ($SVI < 50$ mL/g, MLSS $= 3{,}778$ mg/L) that also revealed a P-metabolism typical for the PAO-phenotype; an enunciated anaerobic

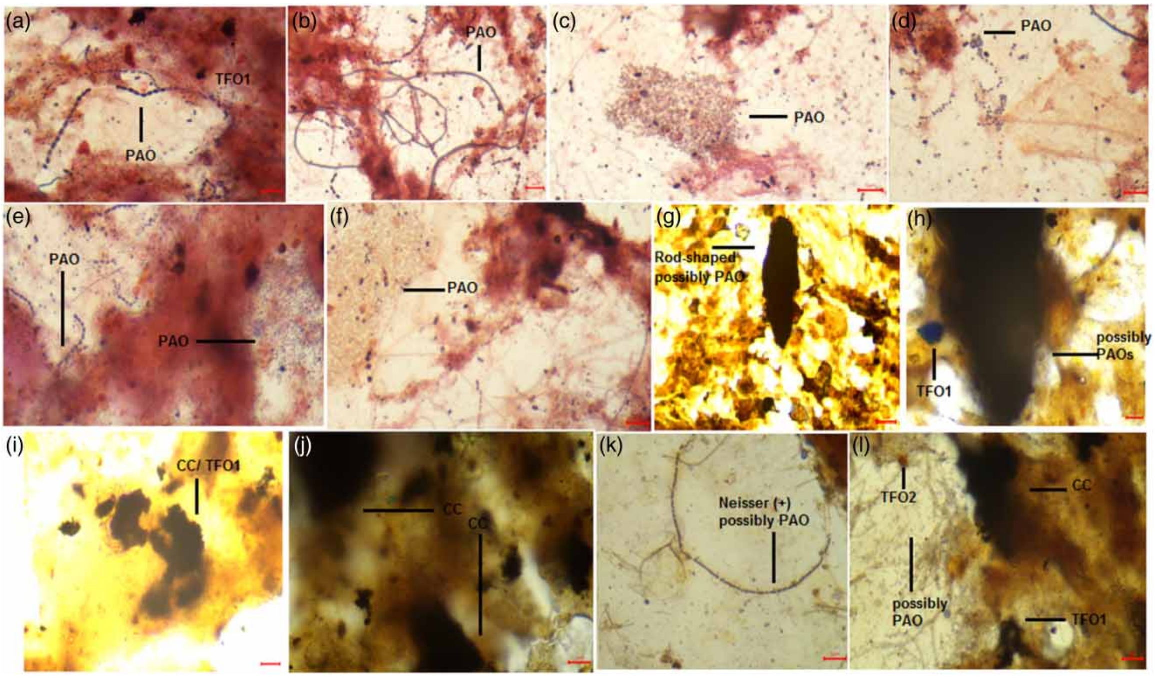


Figure 6 | Bright-field micrographs of samples from Mumbai SBR (with selector) biomass: Case I. Panels [a], [b], [c], [d], [e], and [f]; end of the anaerobic period. Panels [g] to [l]; end of the aerobic period. Samples in panels [g], [h], [i], [j], [k] and [l] were examined with Neisser-stain for poly-P images and samples in panels [a], [b], [c], [d], [e] and [f] were examined with Sudan Black B for PHB inclusions. The description of these micrographs is summarized in Table 5. Panels [a], [b], [d], and [e] show PHB granules in the filaments and [c], [d], [e], and [f] show PHB granules in floc-formers. Panels [g] to [l] are showing poly-P globules possibly indicating CC, TFOs, and PAOs, where [k] shows possible PAOs in the filaments and the panels [g] and [h] are displaying the same rod-shaped cell (PAO) at different magnifications (10X and 100X respectively). The size of PHB granules varied in these samples, the size of granule in panel [a] was bigger and observed as 0.2–0.5 μm, and the granules [b], [d], and [e] (filaments) are 0.1–0.3 μm whereas granules in panels [c], [e] and [f] (floc-formers) are lesser in size (<1 μm). The causes of these variations could be the difference in morphology of PAOs or the capacity of PHB granule (cluster) formation by PAOs. Floc-formers are more than filaments, and these filaments are helpful/reflecting positive results for EBPR. Black-colored stains appear due to either longer exposure time of crystal violet and methylene blue solution or higher % of TP (as poly-P) in sludge biomass.

Table 5 | Brief description of micrographs shown in Figure 6

Figure 6	Morphology	End of anaerobic period	End of aerobic period	Observation	Abbreviation
Panels (A to H, K, L)	Rod-shaped cells	P (−−−) PHB (++−)	P (++−) PHB (+−−)	Rod-shaped cells are Neisser positive and PHB positive (anaerobic condition: PHB more; poly-P less and aerobic condition: PHB less; poly-P more) – possibly PAOs (P)	PAO
Panels (H, I, L)	Tetrads/ Sarcina-like cells	eP (+−−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – possibly GAOs (eP)	TFO1
Panels (L)	Tetrads/ Sarcina-like cells	eP (−−−) PHB (+−−) Gram (−−−)	eP (−−−) PHB (−−−) Gram (−−−)	Do not stain at all, gram-negative – possibly GAOs (eP)	TFO2
Panels (I, J, L)	Coccoid-clusters	eP (++−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – GAOs, possible ordinary facultative heterotrophs (OHOs), denitrifiers, or even nitrifiers (eP)	CC

P-release in the anaerobic selector (32.9 mg/L or 9 times the influent) followed by a noteworthy aerobic P-uptake of 35.4 mg/L in the aeration tank (~81.8% uptake) (Dulekgurgen *et al.* 2011; Srivastava *et al.* 2021).

2. Similarly, in the Mumbai SBR plant, most of the bright-field micrographs (panels A to H, K, and L) gave shreds of evidence of PAOs community, and the structures of TFOs and CCs resembled GAOs, OHOs, nitrifiers, and

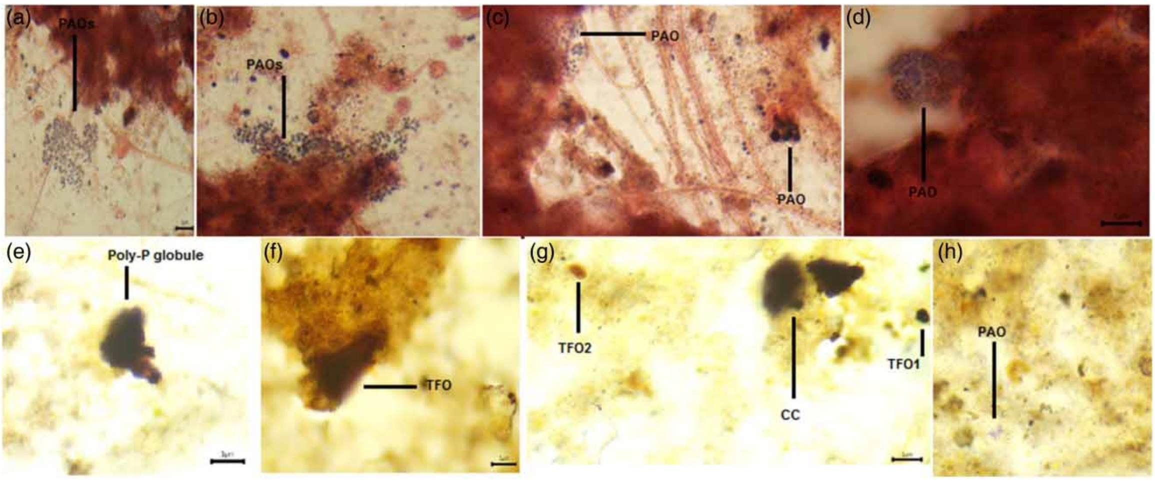


Figure 7 | Bright-field micrographs of samples from Gurgaon SBR (without selector) biomass: Case I. Panels [a], [b], [c], and [d]; end of the anaerobic period. Panels [e], [f], [g] and [h]; end of the aerobic period. Samples in panels [e], [f], [g], and [h] were treated with Neisser-stain for poly-P visualization and in panels [a], [b], [c], and [d] were treated with Sudan Black B for PHB staining. Analysis of these micrographs is summarized in Table 6.

Table 6 | Brief description and interpretation of micrographs shown in Figure 7

Figure 7	Morphology	End of anaerobic period	End of aerobic period	Observation	Abbreviation
Panels (A, B, C, D, H)	Rod-shaped cells	P (−−−) PHB (++−)	P (++−) PHB (+−−)	Rod-shaped cells are Neisser positive and PHB positive (anaerobic condition: PHB more; poly-P less and aerobic condition: PHB less; poly-P more) – possibly PAOs (P)	PAO
Panels (F, G)	Tetrads/ Sarcina-like cells	eP (+−−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – possibly GAOs (eP)	TFO1
Panels (G)	Tetrads/ Sarcina-like cells	eP (−−−) PHB (+−−) Gram (−−−)	eP (−−−) PHB (−−−) Gram (−−−)	Do not stain at all, gram negative – possibly GAOs (eP)	TFO2
Panels (G)	Coccoid-clusters	eP (++−) PHB (−−−)	eP (++−) PHB (−−−)	It stains at their cell walls only, so their PHB is negative – GAOs, ordinary facultative heterotrophs (OHOs), denitrifiers, or even nitrifiers (eP)	CC

denitrifiers in the panels H, I, J, and L (Dulekgurgen *et al.* 2003, 2011). As per Table 1, EBPR was high in the plant, and nitrogen removal was excellent. The readily available carbon source in the wastewater; that is, rbCOD was observed as 32% of total COD, which might have provided enough VFAs (longer sewer lines have credited/supplemented the VFAs) and significant formation of PHBs in the anaerobic phase for the effective proliferation of PAOs to have occurred (Broughton *et al.* 2008). The poly-P content as TP% in the sludge was also the highest (5–7), and the operational design parameters (DO, ORP, pH, sludge recycle rates, and anaerobic HRT) were managed in the plant concerning the biological nutrient removal approach.

3. Gurgaon plant's sludge morphology and micrographs represented a diverse community of PAOs, TFOs (as GAOs), and OHOs. EBPR was excellent in the plant (>20%), but the relative rates of nitrification (50%) were less and didn't even satisfy the latest TN effluent standards of 10 mg/L.
4. In contrast, Roorkee and Salori plants were mainly TN removing plants, but some EBPR (6%–14%) was observed. They had a diverse community of some PAOs, GAOs, OHOs, nitrifiers, and denitrifiers, which

can be interpreted from the bright-field micrographs and Tables 2 and 3, respectively. >95%, >97%, >94%, >87%, and >93% organic matter removal in Roorkee, Salori, Varanasi, Mumbai, and Gurgaon plants, respectively, was believed to be the contribution/involvement of ordinary heterotrophs, consuming rbCOD (or sBOD) that remained at the aerobic phase (Dulekgurgen *et al.* 2003).

5. Novel findings of the research include: (a) at rbCOD: TP of 12–19, BOD/TP $\geq$ 20, COD/TP $>$ 45, TP% in sludge $>$ 4 and sufficient PHB formation in the anaerobic zone and positive Neisser staining results in the aerobic sludge signify good EBPR occurrence (>20%) in full-scale SBR plants (as observed in the SBR plants of Varanasi, Mumbai, and Gurgaon); (b) higher rbCOD fraction in COD of >9, BOD/TKN $>$ 4 and COD/TN of at least five may contribute to higher SND% (75.5–98.5) in the SBR plants; however, anoxic/anaerobic selectors are considered equally essential for simultaneous nutrient removal, and SBR plants equipped with selector zones behave better than non-selector attached SBRs, i.e., Gurgaon plant; (c) appearance of OHOs in aerobic sludge is perceived to contribute to higher organic matter removal in SBR plants; (d) for higher EBPR with effective nitrogen removal succinctly rbCOD/COD (>9), C/N (>5), and rbCOD/TP (10–20) can be considered necessary along with pre-anoxic/anaerobic selector zone conditions (ORP, DO-control, pH, HRT, and sludge recycling rates) and diverse microbiome in the sludge (PAOs and OHOs consisting of nitrifiers and denitrifiers); (e) the comprehensive methodology can open several microbiological perspectives with the biochemical performance of the SBR plants that can ultimately help in relating with quantitative microbial analysis in the future.

Uncertainties and implication

The Varanasi, Mumbai, and Gurgaon situated SBR plants performed satisfactorily in achieving >75% TP removal efficiencies. Even though TFOs observed in this study are similar to GAOs morphologically, their exact negative result during PHB staining in the anaerobic period requires inquiry into their role and identity as GAOs. The appearance of some non-Poly P bacteria in the plants obscures the determination of the process concerning the calculated parameters of the EBPR mechanism. The microbiological interrelations between the PAOs and the remaining microflora and the extent of contribution of each group to the observed EBPR performance remain incomprehensible. The results justified that these EBPR systems should be analyzed further concerning the microbial diversity and population composition through advanced molecular biological techniques and conventional microbiological and biochemical characteristics to facilitate and elucidate the uniqueness, purpose, and involvement of various groups in the EBPR sludge.

CONCLUSIONS

The microbial community structure of the enhanced biomass achieved in SBR plants was confirmed to be very diverse concerning morphology and function. These consequences are in acceptable agreement with microbiological interpretation and phylogenetic analysis of different researchers. Sustaining partial or complete EBPR systems with a granular flocculated texture was possible in the plants; however, particularly phosphorus, together with nitrogen and carbon removal efficiencies, were magnificently influenced by influent characteristics as of COD/TP and rbCOD/TP ratio, the extent of negative ORP in the anaerobic compartments of a selector/or anaerobic phase of treatment and their retention times, longer sewer lines before reaching wastewater pumping stations to produce sufficient VFAs, controlled DO and OUR operations, and minimum recycle of nitrates in the anaerobic zones. The analysis in this study may better optimize the systems for EBPR simultaneously with nitrogen removal and can open the novel perspectives of microbiology connected with EBPR systems through qualitative and quantitative approaches.

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DISCLOSURE STATEMENT

The authors declare no competing interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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