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(Article begins on next page)

1 **Containment of a genetically modified microorganism by an**
2 **activated sludge system**

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1 **Abstract**

2 The effectiveness of physical, chemical and biological barriers to the diffusion of genetically modified
3 microorganisms (GMMs) to prevent their release into the environment is currently under scrutiny
4 worldwide because of the associated potential ecological impacts. An industrial discharge of a non-
5 sterilized fermentation broth containing GMM biomass into a conventional municipal wastewater
6 treatment plant would deliver the GMMs into the activated sludge system process (ASSP). The present
7 work aimed to model and evaluate the containment capability of a small ASSP (part of a 20,000 people
8 equivalent municipal plant) in the event of receiving GMM biomass from a medium-small
9 biotechnological plant dedicated to the production of polyhydroxyalkanoates (3,000 ton/year of
10 biopolymer). An actual GMM (*Pseudomonas putida* KTOY06) was injected into a bench-scale ASSP
11 (ASSP_{Lab}) in a quantity proportional to the relative dimensions of the plants mentioned. The
12 experimental and model results indicated that the ASSP of the target municipal treatment plant would
13 not be capable of holding back such a sudden input of GMM; 6 hours after the discharge, 11-15 % of
14 injected GMM cells were released through the clarified stream of the ASSP_{Lab}, with the rest being
15 gradually released over time. Since the GMM employed did not exhibit any growth in the ASSP_{Lab}, its
16 concentration in the clarified water stream would not represent a substantial risk of release into the
17 environment if appropriate tertiary treatment were integrated. This study confirmed the necessity of a
18 thorough risk assessment of biotechnological processes prior to their implementation.

19

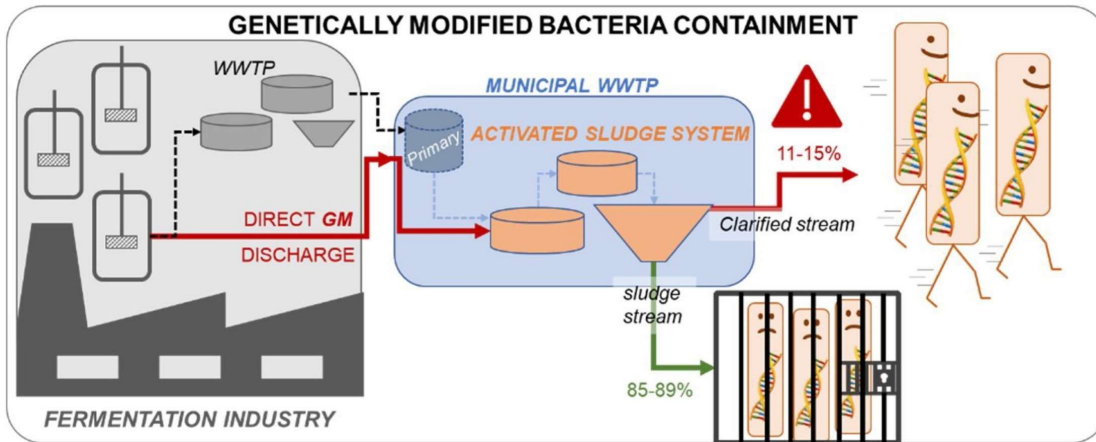
20 **Keywords**

21 GMM fate; industrial effluent; fermentation safety; modelling; tertiary treatment; municipal wastewater
22 treatment plant

23

24

1 GRAPHICAL ABSTRACT



2

1 **Highlights**

- 2 • GMM-containment by small municipal activated sludge systems was studied at bench-scale
- 3 • Mass balance model with survival and settling terms fit the experimental results
- 4 • 11-15% of the GMM would be released alive through the municipal secondary treatment
- 5 • Municipal tertiary treatment becomes particularly important as to avoid GMM release

1 **Introduction**

2 Industrial biotechnology increasingly represents a route for sustainable manufacturing and waste
3 valorisation [1,2]. Many molecules are produced on an industrial scale through biotechnological
4 methods, *inter alia* pure culture fermentations and/or biotransformations for vaccines, building blocks
5 and biopolymers. 'White biotechnology' often employs genetically modified microorganisms (GMMs),
6 which offer the possibility of lower production costs [3]. Due to this increasing practice, public concern
7 on commercial utilization of GMMs has been increasing, e.g. realizing the need to identify potential
8 hazards and to employ risk assessments and controls. Most industrial strains are GRAS (generally
9 recognised as safe) microorganisms, chosen to avoid toxicity or pathogenicity and tailored or modified
10 for enhanced productivity under process conditions, which in turn may render the GMMs poor
11 competitors with wild type strains occurring outside the fermenter [4]. Nevertheless, these premises do
12 not consider that a released GMM may survive long enough to transfer genes to environmental strains,
13 potentially priming a series of consequences that can be summarized as 'environmental
14 destabilization'. There are reports demonstrating that GMM strains can survive in the environment [5–
15 7] and, when thriving in an external environment, were shown to overcome engineered biological
16 containment measures [8–10] and to confer mutant characteristics on wild type microorganisms
17 through horizontal gene transfer [11,12].

18
19 According to the EU regulatory framework on contained use of GMMs (European Directive
20 2009/41/EC, [13]) all appropriate measures should be taken to avoid adverse effects on human health
21 and the environment, through preliminary assessments of the risks that it may pose. This can be
22 targeted through physical containment, or a combination of technical tools and dedicated protocols.
23 The importance of the latter becomes even more crucial when considering that an estimated 68% of
24 failures are due to human error [14]. The main topic for the safety issue is the physical containment of

1 GMMs and the directive provides the measures to be adopted. Among them, inactivation of GMMs by
2 validated methods is provided within the information about accident prevention and emergency
3 response plans. It takes into account contaminated materials and waste, including those occurring in
4 process effluents before final discharge (Annex IV-Table II of 2009/41/EC) and describes the
5 procedures and plans for verification of the continuing effectiveness of the containment measures
6 (Annex V- Part C). For industry, this requirement entails the obligation to incorporate biological
7 environmental monitoring into its working practices. However, the same EU directive indicates that if
8 the GMM is a derivative of a class 1 microorganism, inactivation of the residual effluent containing the
9 GMM is only discretionary. On the other hand, according to the most recent EFSA guidance on the use
10 of GMMs, the environmental impact of any GMM should be considered in terms of its viability and
11 persistence into the environment, as well as the risk of horizontal transfer of genetic traits [15]. Many
12 authors have pointed out that GMM-utilization practices should take into account different kinds of
13 genetic mutation and the assessment of environmental risk before any industrial use, just as for
14 genetically modified organisms (GMOs) [16,17].

15

16 A process risk analysis should also be performed by evaluating the potential release of GMMs into the
17 environment, depending on process features. Industry must first ascertain the nature of the risk
18 associated with its processes and then organize environmental monitoring using appropriate techniques.
19 Such monitoring must not only take into account the information on GMM release into the
20 environment, which is scarce compared with that on the environmental fate of conventional wild-type
21 microorganisms [18], but must also consider the likelihood of accidents occurring due to human error
22 or deliberate misuse, especially during upscaling [19].

23

1 Large-scale fermentations generate high amounts of liquid waste, which must be fed to a wastewater
2 treatment plant (WWTP) to remove organic and nitrogen content before returning the effluent to the
3 water cycle. Such a conventional discharge represents the first biological risk associated with the
4 release of GMMs or their genetic material [20]. Therefore, it is important to provide the public with
5 accurate information about the fate of GMMs release by means of spill or discharge without pre-
6 treatment, so that stakeholders can feel comfortable in accepting such techniques and the resulting
7 products. Few studies have been published on this topic. The first one showing that a recombinant
8 strain of *E. coli* used in the commercial production of bovine somatotropin does not persist in
9 nonsterile water, soil and sewage dates back to 1991 [21]. The same author later reported that cellular
10 concentration decreases by up to 5 orders of magnitude in 147 h [22]. Some years ago the potential fate
11 of a GMM if discharged into a WWTP was reported for the first time [23]. Specifically, in the event of
12 an accidental discharge of a *B. subtilis* fermentation broth, only a small percentage of discharged cells
13 could reach the final unit (settler) of the WWTP. However, the plant described was composed of an up-
14 flow anaerobic reactor, an air-lift reactor and a settler. This configuration does not represent that of a
15 typical municipal WWTP, which includes an activated sludge system process (ASSP) consisting of
16 sequential anoxic (denitrification phase) and aerobic (oxidation phase) conventional open vessels,
17 followed by a clarification step carried out in the settler. These ASSPs employ denitrification and
18 oxidation vessels that are closer to completely mixed reactors rather than to up-flow and air-lift ones.
19 The main goal of the present work was to evaluate the risk of releasing a GMM strain into the
20 environment, in the case where an accidental industrial discharge of a GMM-containing effluent (at
21 high cell density) directly reaches a small conventional municipal WWTP (20,000 people equivalent).
22 A worst-case scenario was considered that the GMM-biomass reached the ASSP without significant
23 modification or dilution. Accordingly, a consistent quantity of a GMM producing
24 polyhydroxyalkanoates (PHAs) was spiked into a bench-scale ASSP to simulate such a release and

1 study GMM fate. The GMM strain and its DNA were monitored through selective-plate counting and
2 quantitative PCR (qPCR), respectively, during the scaled-down experiment so as to determine its
3 persistence and containment within the experimental ASSP.

5 **Materials and Methods**

6 *Microorganism*

7 The GMM used in the present study was *Pseudomonas putida* KTOY06 kindly provided by Prof. Guo
8 Qiang Chen (Centre for Synthetic and Systems Biology, Tsinghua University); it was obtained after
9 deletion of genes involved in the β -oxidation pathway [24] and is a kanamycin and rifampicin resistant
10 strain which was modified for production of PHA homopolymers. The growth of *P. putida* KTOY06
11 was carried out in a 3 L bench-scale bioreactor (Sartorius Biostat B) using LB medium supplemented
12 with glucose (5 g/L). Culture conditions were 30°C, pH 7 and $pO_2 > 30\%$.

14 *Bench-scale activated sludge system and start-up*

15 A conventional ASSP (with pre-denitrification) was set up at bench-scale (ASSP_{Lab}) to simulate the
16 biological unit of a municipal WWTP.[25] It consisted of three vessels, namely the anoxic reactor
17 (mixed with a stirring bar and through recycling), the aerobic reactor (mixed by supplied air), and the
18 settler. **Figure 1** shows the configuration, working volumes, stream flows and residence times (τ) of the
19 three units. Briefly, the system functioned in continuous mode, with an incoming flow rate (F_1) of 330
20 mL/h and two recycle lines (F_4 and F_6). Sludge age was fixed at 1 week by discharge through the settler
21 purge line (F_7), with a flow rate of 12 mL/h.[26,27]

22 The ASSP_{Lab} start-up was carried out by filling all vessels (100% v/v) with sludge collected from the
23 municipal WWTP of Trebbo (Bologna, Italy). Thus, the ASSP_{Lab} contained the microbiome and flocs
24 occurring in an actual ASSP. Thereafter, the ASSP_{Lab} was fed with a lab-prepared solution that

1 simulated the soluble fraction of a real influent of a municipal WWTP [26,28]. It was composed of
2 (g/L): CH₃COONa (0.210), yeast extract (0.237), glucose (0.217), NH₄Cl (0.278), Na₂CO₃ (0.1),
3 MgSO₄·7H₂O (0.06), CaCl₂·2H₂O (0.009), K₂HPO₄ (0.004), and micronutrients solution (0.4 mL/L).
4 This last was composed of (g/L): FeCl₃·6H₂O (1.5), H₃BO₃ (0.15), CuSO₄·5H₂O (0.03), KI (0.18),
5 MnCl₂·4H₂O (0.12), Na₂MoO₄·2H₂O (0.06), ZnSO₄·7H₂O (0.12), CoCl₂·6H₂O (0.15) and EDTA
6 (0.01).

7

8 ***Experimental approach***

9 The aim was to simulate and study the discharge of a GMM-fermentation broth from a typical
10 industrial biotech facility into a small municipal WWTP (20,000 p.e.). A reasonable “worst-case
11 scenario” was considered by assuming: (i) an instantaneous full discharge of GMM-fermentation broth
12 at industrial volume and concentration (*ca.* 100 g/L); (ii) that the broth was not pre-treated (*e.g.* by heat
13 or chemical sterilization); and (iii) that the GMM biomass reached the ASSP without being degraded,
14 retained, diluted or modified by up-stream processes (including primary physical treatments at the
15 WWTP).

16 The experimental fermentation broth volume to be “discharged” was determined by considering the
17 expected worldwide PHAs production capacity (240,000 tonnes/year for 2030) [29] and the Italian to
18 world population ratio (*ca.* 8.2×10^{-3}); a potential PHAs production plant covering the Italian market
19 would have a capacity of *ca.* 3,000 tonnes/year. Thus, a process plant with dimensions described
20 elsewhere [30] for the same annual production was taken into consideration to calculate that 228 m³ of
21 culture broth, containing 100 g/L of grown cells holding 77% of PHAs, could potentially be released
22 into the target WWTP. An Italian WWTP for 20,000 person equivalents would have a 10,000 m³
23 anoxic vessel with *ca.* 6 g/L of suspended sludge.[26,31] Therefore, assuming an instantaneous GMM
24 point feeding (*i.e.* full discharge), the ratio between the suspended-biomass contained in the latter

1 vessel and the GMM-biomass entering is *ca.* 2.4 (60,000 kg of sludge/25,000 kg of GMM-biomass).
2 Since the ASSP_{Lab} had a 1 L-anoxic vessel holding *ca.* 5 g/L of suspended sludge, 2.1 g of GMM-
3 biomass were fed. For this, 500 mL of *P. putida* KTOY06 culture grown to 4.2 g/L under optimal
4 conditions (above) were centrifuged (8°C, 6,797 rcf for 15 min) and the obtained pellet was suspended
5 in 21 mL of mineral medium. It was concentrated to 100 g/L using a different culture medium (rather
6 than LB medium) in order to obtain a more representative “industrial discharge” to be added into the
7 anoxic chamber.
8 The ASSP_{Lab} had been working for 5 days prior to the GMM-injection, allowing its performance to be
9 verified (*e.g.* denitrification, COD reduction, etc.) under steady state conditions. Thereafter, all flows
10 were temporarily stopped, the GMM-biomass was injected into the anoxic chamber, followed by
11 stirring for 10 min to produce homogeneity of the exogenous cells within the indigenous sludge, and
12 the pumps were restarted. To monitor the ASSP_{Lab}, 5 mL samples were withdrawn at -24, -2, 0
13 (injection time), 20', 40', 1, 1.75, 2.5, 4, 6, 11, 19, 24, 48 and 72 h from the anoxic and aerobic
14 chambers and the settler sludge, while 50 mL were sampled from the settler supernatant and the
15 accumulated-clarified effluent. After each sampling, the samples were divided into three parts: one
16 portion was used immediately for cell counting (see below); a second was mixed with ethanol (50%
17 v/v) and stored at -20 °C for DNA extraction and qPCR analysis (see below); and the rest was directly
18 stored at -20 °C until chemical analyses were performed (see below)

19

20 ***Modelling the fate of the GMM***

21 To evaluate the fate of the GMM in the ASSP_{Lab}, the following differential equations describing mass
22 balances around each chamber were used:

23 - anaerobic vessel (Eq. 1):

$$24 \quad \frac{dC_{An}}{dt} * V_{An} = F_1 * C_{Stu}(t) + F_3 * C_{Ar}(t) - F_2 * C_{An}(t) + \mu_{res; An} * C_{An}(t) * V_{An}$$

1 - aerobic vessel (Eq. 2):

$$2 \quad \frac{dC_{Ar}}{dt} * V_{Ar} = F_2 * C_{An}(t) - F_2 * C_{Ar}(t) + \mu_{res;Ar} * C_{Ar}(t) * V_{Ar}$$

3 - settler, for the clarified zone (Eq. 3):

$$4 \quad \frac{dC_{Cla}}{dt} * (V_S * (1 - i2))$$
$$5 \quad = F_3 * C_{AR}(t) - F_5 * C_{Cla}(t) - F_3 * C_{AR}(t) * i + \mu_{res;Cla} * C_{Cla}(t) * (V_S * (1 - i2))$$

6 - settler, for the sludge-concentrated zone (Eq. 4):

$$7 \quad \frac{dC_{Slu}}{dt} * (V_S * i2) = F_3 * C_{AR}(t) * i - (F_7 + F_1) * C_{Slu}(t) + \mu_{res;Slu} * C_{Slu}(t) * (V_S * i2)$$

8 where F_x , C_x , μ_x and V_x are the flow rates, concentrations, resulting GMM generation rates and vessel
9 volumes presented in **Figure 1**. i and $i2$ represent the mass fraction settled (settled/fed) and the volume
10 fraction of the sludge-concentrated zone (sludge_volume/settler_volume), respectively.

11 Model correctness was verified in a preliminary experiment by injecting an aqueous solution of NaCl
12 (30 g/L) into the ASSP_{Lab} operating with distilled water. The NaCl concentration in each vessel was
13 monitored for 48 h by conductivity measurements. The data were fitted using Matlab tools *fminsearch*
14 and *ode45*. (Supplementary Information, S4)

15

16 ***Analytical procedures***

17 *Quantitative PCR and colonies counting*

18 For molecular analysis, total DNA was extracted from 2 mL of sludge or 25 mL of clarified
19 supernatants (obtained by centrifugation at 10,620 rcf for 10 min) using the PowerSoil® DNA Isolation
20 Kit (MoBio Laboratories, Carlsbad, CA, USA), according to the manufacturer's protocol. Quality of
21 the extracted DNA was checked on agarose gel (1% v/v) and concentration was measured with
22 NanoPhotometer® P-330 (Implen GmbH, Munchen, Germany). Primers 484F and 681R – designed to

1 target specifically the employed GMM through the Δ *fadBA* gene- were used for amplification. The
2 primers were designed in this study, starting from previous descriptions [24] and were tested *in silico*
3 against the *P. putida* KT2440 genome as negative set, resulting in no mismatches (as described in
4 Supplementary Information Section S2.1). The qPCR protocol was previously optimized in order to
5 have an efficiency $\geq 80\%$ in the range of 10^2 to 10^7 copies and the lowest signal for the non-specific
6 amplification of *P. putida* KT2440 used as negative control. The qPCR reactions (20 μ L) contained 1x
7 SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories S.r.l., Milan, Italy),
8 forward and reverse primers 150 nM and 10 ng of DNA template. The temperature program for the
9 qPCR consisted of a step at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 min and 60 °C for
10 20 min. Specificity was checked with a final melt curve stage from 60 to 95°C at increments of 0.3°C.
11 Linear 6-point calibrations in the range of 10^7 to 10^2 copies were obtained by using cloned Δ *fadBA*
12 inserts as described in Supplementary Information Section S2.1; efficiencies ranged from 85 to 99%.
13
14 Plate counting was carried out using selective LB agar plates containing rifampicin and amphotericin B
15 (after cooling) at final concentrations of 500 mg/L and 2.5 mg/L, respectively. Aseptic plating was
16 performed using 30 μ L of samples from serial dilutions. Colonies were counted after incubation at 30
17 °C overnight and concentrations calculated as colony forming units (CFU/mL).
18
19 Preliminary tests showed the qPCR and plate counting assays to be accurate quantification methods.
20 This was validated by analysing different mixtures of *P. putida* KTOY06 and sludge in proportions
21 from 1 (half sludge and half GMM) to 10^7 g_sludge/ g_GMM. Correspondence with expected values
22 was verified: the qPCR was reliable in the range $1-10^5$ (with errors <8%), while the signals obtained for
23 higher ratios were at noise level (assigned to nonspecific amplification). The colony counting method

1 was reliable in the range $1-10^6$ (with errors $<7\%$). Extended description and curves are shown in
2 Supplementary Information S2.2 (Figure S3).

3

4 *Monitoring the ASSP_{Lab} performance*

5 Soluble chemical oxygen demand (COD) was measured with a colorimetric kit (AQUALYTIC

6 Vario MR), using glucose solutions for the calibration. Total nitrogen (TN) was measured

7 spectrophotometrically as described in [32].

8 Ammonium ion concentration was measured by means of a selective electrode (CRISON

9 9663 C) and the results were expressed as ammonia nitrogen (N-NH_4^+). Nitrate and phosphate were

10 measured by ion-exchange chromatography (Dionex DX-120 with a column IonPac®

11 AS14A) as described elsewhere [33]. Prior to analysis, samples were centrifuged (10,620 rcf, 10 min,

12 6°C), filtered ($0.2\ \mu\text{m}$) and diluted as required.

13

14

15 **Results and Discussion**

16 *ASSP_{Lab} assessment*

17 The ASSP_{Lab} was functioning for 5 days before addition of the GMM-biomass, achieving steady-state

18 conditions after 26 h from start-up. **Figure 2** shows the concentration profiles of target chemicals in all

19 the ASSP_{Lab}'s vessels during the experimental period; points at -24 and -2 h were taken before GMM

20 injection (0 h). The GMM addition caused perturbations in the concentration trends (TN, N-NH_4 , P-

21 PO_4 and COD). The ASSP_{Lab} returned to steady state after 5 h (when variations of target chemical

22 parameters were $<10\%$ of the respective mean values) and was monitored over the following 72 h.

23 During the steady state, the ASSP_{Lab} exhibited good performance in terms of TN, P- PO_4 and COD

24 abatement; average falls in concentrations were (%): TN 48.4 ± 5.8 , N-NH_4^+ 65.9 ± 6.2 , P- PO_4 $16.6 \pm$

1 8.3 and COD 65.2 ± 2.8 . These values were consistent with the expected abatements in actual
2 municipal treatment plants operating correctly [26,31]. In addition, monitoring of suspended solids
3 (biomass) and pH confirmed the stability and functionality of the ASSP_{Lab} (**Figure 3**).

4
5 To analyse the fate of the GMM, an ordinary differential equation system was proposed and verified
6 through a preliminary test in which the ASSP_{Lab} (filled and fed with only water) received a single NaCl
7 pulse. During fitting trials, the objective function - to be minimized - was best defined as $(\log C_x - \log$
8 $C_{x, experimental})^2$. The fitting curves (Supplementary information S3, Figure S4) have regression
9 coefficients ≥ 0.93 and the resulting generation rate values ($\mu_{An} = 0.0436 \text{ h}^{-1}$, $\mu_{Ar} = 0.0692 \text{ h}^{-1}$, $\mu_{Cla} = -$
10 0.0575 h^{-1} and $\mu_{Stu} = -0.0083 \text{ h}^{-1}$) are in accordance with the absence of either cell death or growth.

11 *GMM monitoring and fate*

12 In the experiment with the GMM, no CFUs were detected in any of the ASSP_{Lab} compartments before
13 inoculation, whereas the number of recombinant gene copies/mL was consistent with the background
14 noise of the assay: after injection (at time 0) the GMM concentration was $1.04 \times 10^9 (\pm 4.23 \times 10^8)$
15 CFU/mL or $2.26 \times 10^9 (\pm 5.58 \times 10^7)$ copies/mL, whereas the expected value was *circa* 1×10^9
16 inoculated cells/mL. Currently, qPCR assays are still not implemented as compliant by the regulators,
17 as technical procedures are still being standardized (*i.e.* ISO/TS 12869:2012), but most recent safety
18 guidelines concerning release of GMMs into the environment require determination of the amounts of
19 recombinant DNA rather than whole cells[35]. Many qPCR limitations, such as background signal or
20 possible detection of dead cells, can be easily overcome by using modifications, such as different DNA
21 extraction methods or PMA-qPCR [36], respectively. In this study, the plating method yielded a lower
22 limit of detection than qPCR, probably due to the peculiar nature of the sludge used. Hence, plate
23 counting (CFU/mL) was used to model the fate of the GMM injected in the ASSP_{Lab}.

24

1 The following initial conditions and rules were assessed: *i*) only the initial conditions for $C_{Ar}(0)$, C_{Cla}
2 (0) and $C_{Slu}(0)$ were fixed as zero value, while $C_{An}(0)$ could be in the range of its average
3 experimental value with the respective standard deviation ($6.58 \times 10^8 \leq C_{An}(0) \leq 1.03 \times 10^9$ CFU/mL); *ii*)
4 the GMM could settle or not, this determining the constraint $0.5 \leq i \leq 1$; *iii*) since the volume of the
5 concentrated sludge in the settler was observed to vary between 0.45 and 0.5 L, the constraint $0.45 \leq i_2$
6 ≤ 0.50 was imposed. The resulting semilogarithmic concentration trends fit the experimental results
7 very efficiently, with the following regression coefficients: $R_{An}^2 = 0.81$; $R_{Ar}^2 = 0.99$; $R_{Cla}^2 = 0.85$;
8 $R_{Slu}^2 = 0.96$. All the resulting GMM generation rates were low absolute values ($\mu_{An} = -0.0832 \text{ h}^{-1}$, μ_{Ar}
9 $= -0.0383 \text{ h}^{-1}$, $\mu_{Cla} = 0.1117 \text{ h}^{-1}$ and $\mu_{Slu} = 0.083 \text{ h}^{-1}$), as were those obtained for the NaCl injection,
10 meaning that no significant GMM growth or death occurred in the ASSP_{Lab}. Only the GMM generation
11 rate corresponding to the clarified zone was slightly higher than expected. This was considered a
12 consequence of the sampling procedure for the settler-vessel since decanted-sludge was inevitably
13 slightly resuspended when using the sampling pipette. The experimental and modelled results were
14 transformed into totalities along the experimental time, namely: total GMM contained in the ASSP_{Lab},
15 quantity discharged by the clarified stream and amount eliminated by the concentrated sludge purge
16 (**Figure 4A**). The same fitting curves are shown with the qPCR results in Figure 4B, where highly
17 similar behaviours were detected. This implies that the number of mutant gene traits detected inside,
18 and released by, the ASSP_{Lab} was consistent with the number of CFU, thus indicating that GMM cells
19 were mostly alive at the time of release and that no cell or DNA depletion occurred inside the ASSP_{Lab}.
20 This is again concerning in terms of ecological impact, because the viability of this GMM strain has not
21 been tested in environment-like conditions so far; a gradual input of a determinate species into an
22 ecological niche increases the genetic frequency of particular gene traits, altering the gene pool, even if
23 those traits have low fitness. In contrast to a report [23] for a different type of treatment plant (as
24 mentioned above), the GMM reached the settler vessel 20 min after injection. Moreover, a significant

1 amount of GMM was discharged through the clarified stream after 6 h, both according to the
2 experimental (15 ± 5 %) and modelled (11%) results. At that time, 76 ± 20 % of the initially injected
3 CFUs were still in the ASSP_{Lab} while only 1-2 % were discharged through the concentrated sludge
4 purge-stream.

5 The latter stream might represent a potential hazard, since statistics reported elsewhere [37] indicate
6 that 50.5% of total sewage sludge generated in the EU15 is used in agriculture, while landfill and
7 compost routes account for another 16.4% of the sludge use. Only Germany, The Netherlands, Austria
8 and Belgium use incineration as the main disposal approach, accounting for 26.1% of total EU15
9 generated sewage sludge.

10 *Effluent disinfection*

11 The clarified stream is usually sent to tertiary treatment (*i.e.* free available chlorine (FAC),
12 monochloramine (NH₂Cl), ozone (O₃), chlorine dioxide (ClO₂), and UV light-based strategies, used
13 alone or in combination) to disinfect the water. Hence, this step becomes important to avoid the leakage
14 of GMMs to the environment. This would not only deplete the viable GMM cells released from the
15 WWTP, but would also notably lower the concentration of the mutant gene traits available for
16 horizontal transfer [38]. Thus, further investigation should be carried out in order to determine the
17 possible effects of the tertiary treatment on GMM containment. For example, it would be interesting to
18 assess the depletion rate of the recombinant DNA at that stage to ascertain that, although GMMs might
19 not be contained by the ASSP, the genetic material would hardly pass through it. Here, a preliminary
20 hypochlorite-disinfection test was performed on the clarified ASSP_{Lab} effluent in order to verify the
21 abatement of living GMMs (CFUs). A portion of the collected clarified effluent was batch-treated with
22 a commercial 4.5% NaOCl solution (2 mg/L final concentration) for 20 min, simulating continuous
23 disinfection. The GMM cells, the initial concentration of which was $2.56 \times 10^7 \pm 4.72 \times 10^6$ CFU/mL,

1 were completely abated. The same test was carried out on the fully grown GMM culture; in this case
2 the GMM concentration was reduced from $1.50 \times 10^7 \pm 2.89 \times 10^6$ to 6.67×10^1 CFU/mL.

3 *Risk assessment*

4 The worst-case scenario of a pulse/spike GMM discharge was considered in order to assess the
5 containment capability of a municipal ASSP in the event of an exceptional release. An actual industrial
6 release would last hours instead of seconds (considering a discharge of the 228m³ fermenter
7 hypothesised and described in the Experimental Approach section), and the GMMs would be diluted in
8 the sewer-line with the pre-existing matrix (instead of directly entering the WWTP). This could result
9 in a better mixing of GMMs with the whole WWTP-influent and sludge already in the ASSP, limited
10 retention time variation in each ASSP-chambers and consequently better GMM-containment. On the
11 other hand, to evaluate potential adaptation of the GMMs to the new environmental conditions, a
12 worst-case scenario could also consider continuous discharge (deliberate or unintentional), or extending
13 the experimental evaluation time to increase the probability of GMM adaptation to ASSP conditions.
14 The GMM *P. putida* KTOY06 used in this study did not proliferate throughout the experiment. To
15 extend the experimental time, the employment of actual wastewater (to feed the ASSP_{Lab}) is
16 recommended to diminish/avoid effects on sludge features. Indeed, it is important to consider possible
17 limitations of using a synthetic feeding solution – carrying neither naturally occurring microorganism
18 nor suspended solids - during the experiment. This may influence two aspects, namely: *i*) the
19 minimization of GMM-bacteria predation by protozoa; and *ii*) incorporation of GMM-cells in flocs.
20 The former might overestimate the spiked GMM not contained by the ASSP_{Lab}. This fits with the
21 above “worst case scenario”. For the second issue, the limited flocculation due to the lack of suspended
22 solids in the feed is considered to have negligible impact on influencing GMM containment by the
23 system, considering the flocs occurring in the actual sludge used to start-up the ASSP_{Lab} (see Bench-
24 scale activated sludge system and its start-up). Moreover, since the sedimentation efficiency did not

1 significantly change during the entire experiment (Figure 3), nor the sludge aspect for the first 48
2 hours, it could be inferred that feeding with synthetic wastewater did not alter significantly the original
3 sludge characteristics and behaviour during the short period of the study. Moreover, after 24 h of
4 injection - during which potential sludge alteration was even less - the GMM concentration in the
5 ASSP_{Lab} diminished to *ca.* 1/10 of the injected amount (Figure 4) by leaving the system through the
6 clarified effluent or concentrated-sludge-purge streams (F₅ and F₇). Notwithstanding the potential
7 experimental limitations, which could be more representative of a worst-case scenario, the results
8 support the importance of risk assessment of GMM release outside biotech facilities.

9

10 In conclusion, to effectively prevent such a release into the environment, it is strongly recommended
11 that biotechnology plants implement protocols for the immediate alert of accidental discharges to
12 municipal WWTP managers in order to allow the necessary adjustment (*i.e.* intensification) of the
13 tertiary treatment.

14

15 **Conclusion**

16 A bench-scale activated sludge system (ASSP_{Lab}) was set-up to simulate an industrial discharge
17 containing GMMs into a conventional municipal WWTP, to study its containment potential.

18 The results showed that if a PHA-producing GMM reached the ASSP of a small municipal WWTP
19 (20,000 PE), they would be released into the environment through the clarified stream rather than being
20 contained. Hence, tertiary treatment becomes particularly important in order to avoid such release.

21

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4

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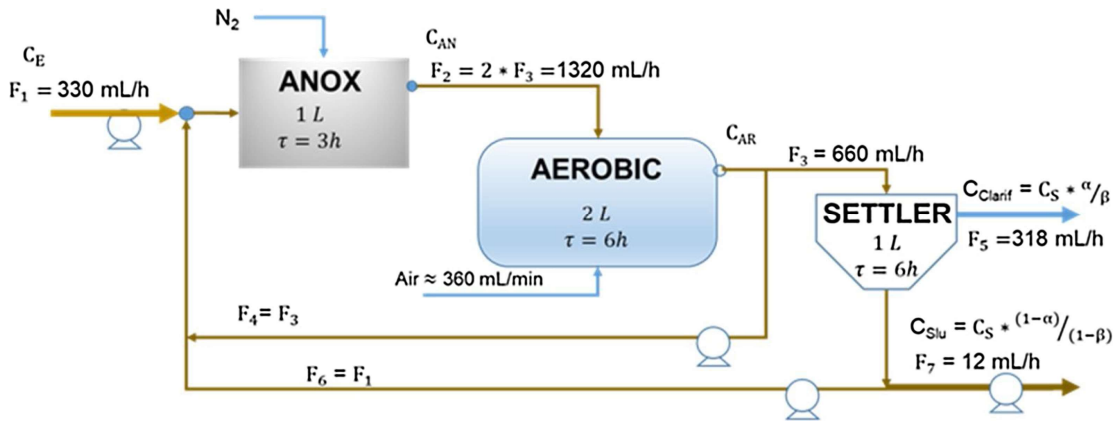
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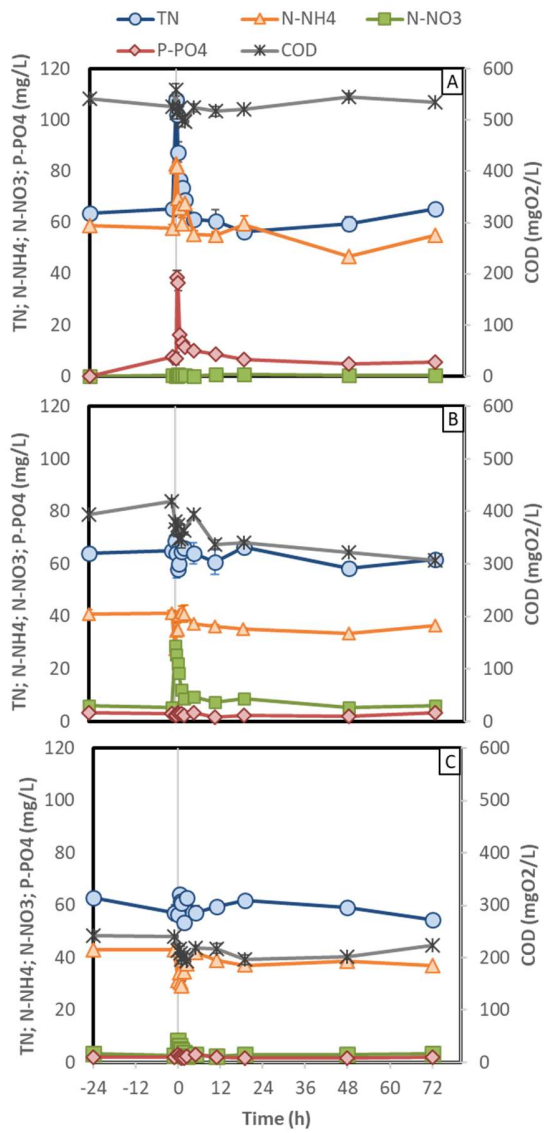
1 **FIGURE and LEGENDS**



2

3 **Figure 1:** ASSP_{Lab} configuration and set up. Flows (F_n) and GMM concentrations variables for the
4 anoxic reactor (C_{AN}), aerobic reactor (C_{AR}) clarified effluent (C_{Clarif}) and concentrated sludge (C_{Stu}) are
5 shown.

1



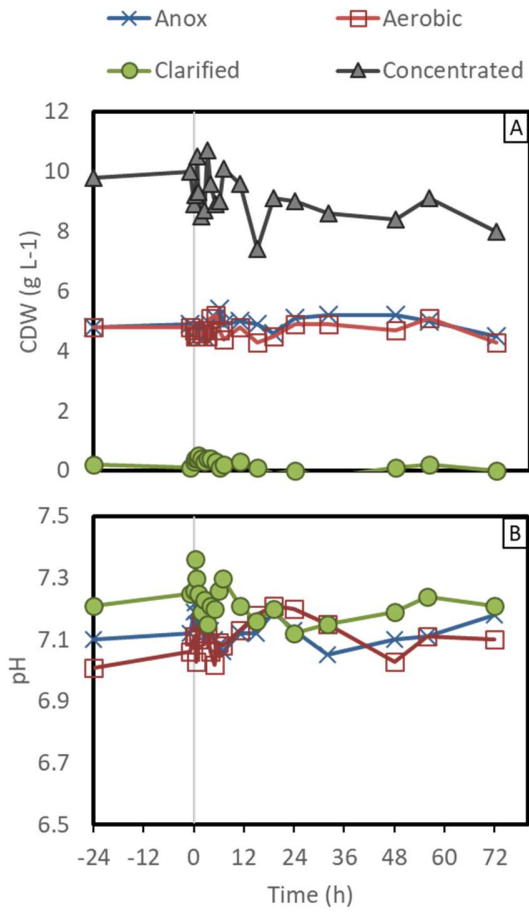
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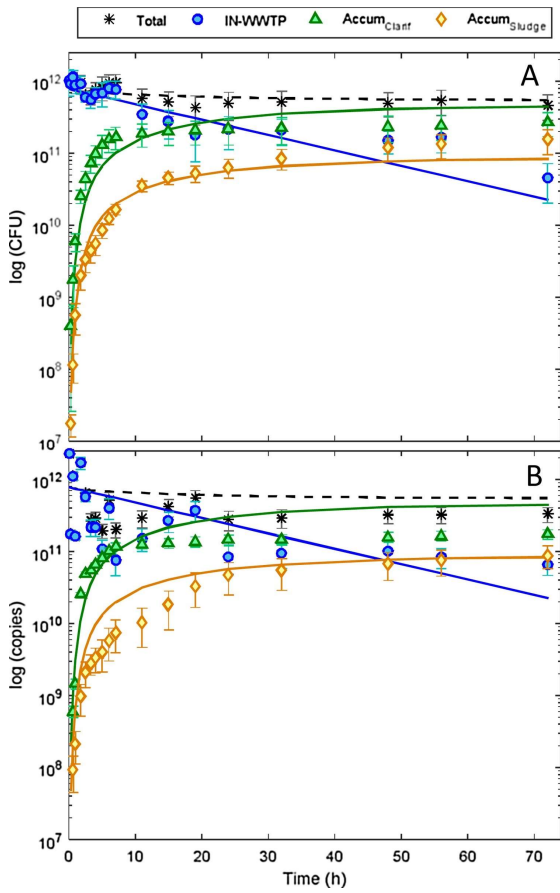
5 **Figure 2:** ASSP_{Lab} performances. Concentration trends during the experiment in the anoxic (A) and
6 aerobic (B) vessels, and in the clarified effluent (C).

7



1 **Figure 3:** ASPP_{Lab} performances in terms of suspended biomass concentration (A) and pH (B) in the
 2 anaerobic and aerobic vessels and in the clarified and concentrated streams.

3



1

2 **Figure 4:** Fate of the GMM after being injected in the ASSP_{Lab}. Total amount of cells quantified along
 3 the experimental time by plate counting (A) and qPCR (B). Measurements allowed to quantify the
 4 GMM: inside the ASSP_{Lab} (IN-WWTP), discharged through the clarified and purged streams
 5 (Accum_{Clarif} and Accum_{Sludge}), and the sum of these (Total).