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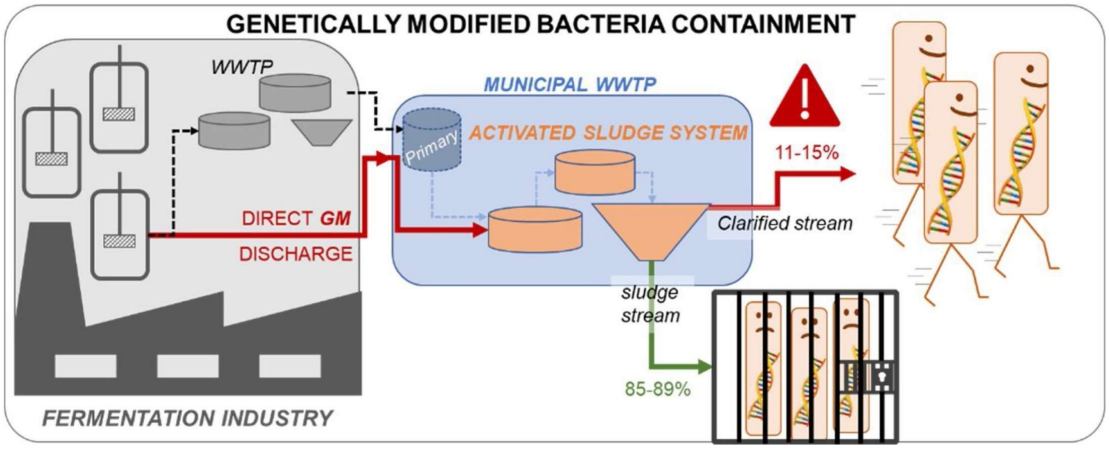
Abstract

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

Keywords

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

GRAPHICAL ABSTRACT



Highlights

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

Introduction

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

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

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

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

Large-scale fermentations generate high amounts of liquid waste, which must be fed to a wastewater treatment plant (WWTP) to remove organic and nitrogen content before returning the effluent to the water cycle. Such a conventional discharge represents the first biological risk associated with the release of GMMs or their genetic material [20]. Therefore, it is important to provide the public with accurate information about the fate of GMMs release by means of spill or discharge without pre-treatment, so that stakeholders can feel comfortable in accepting such techniques and the resulting products. Few studies have been published on this topic. The first one showing that a recombinant strain of *E. coli* used in the commercial production of bovine somatotropin does not persist in nonsterile water, soil and sewage dates back to 1991 [21]. The same author later reported that cellular concentration decreases by up to 5 orders of magnitude in 147 h [22]. Some years ago the potential fate of a GMM if discharged into a WWTP was reported for the first time [23]. Specifically, in the event of an accidental discharge of a *B. subtilis* fermentation broth, only a small percentage of discharged cells could reach the final unit (settler) of the WWTP. However, the plant described was composed of an up-flow anaerobic reactor, an air-lift reactor and a settler. This configuration does not represent that of a typical municipal WWTP, which includes an activated sludge system process (ASSP) consisting of sequential anoxic (denitrification phase) and aerobic (oxidation phase) conventional open vessels, followed by a clarification step carried out in the settler. These ASSPs employ denitrification and oxidation vessels that are closer to completely mixed reactors rather than to up-flow and air-lift ones.

The main goal of the present work was to evaluate the risk of releasing a GMM strain into the environment, in the case where an accidental industrial discharge of a GMM-containing effluent (at high cell density) directly reaches a small conventional municipal WWTP (20,000 people equivalent). A worst-case scenario was considered that the GMM-biomass reached the ASSP without significant modification or dilution. Accordingly, a consistent quantity of a GMM producing polyhydroxyalkanoates (PHAs) was spiked into a bench-scale ASSP to simulate such a release and

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

Materials and Methods

Microorganism

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

Bench-scale activated sludge system and start-up

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

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

simulated the soluble fraction of a real influent of a municipal WWTP [26,28]. It was composed of (g/L): CH_3COONa (0.210), yeast extract (0.237), glucose (0.217), NH_4Cl (0.278), Na_2CO_3 (0.1), $MgSO_4.7H_2O$ (0.06), $CaCl_2.2H_2O$ (0.009), K_2HPO_4 (0.004), and micronutrients solution (0.4 mL/L). This last was composed of (g/L): $FeCl_3.6H_2O$ (1.5), H_3BO_3 (0.15), $CuSO_4.5H_2O$ (0.03), KI (0.18), $MnCl_2.4H_2O$ (0.12), $Na_2MoO_4.2H_2O$ (0.06), $ZnSO_4.7H_2O$ (0.12), $CoCl_2.6H_2O$ (0.15) and EDTA (0.01).

Experimental approach

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

The experimental fermentation broth volume to be "discharged" was determined by considering the expected worldwide PHAs production capacity (240,000 tonnes/year for 2030) [29] and the Italian to world population ratio (*ca.* $8.2x10^{-3}$); a potential PHAs production plant covering the Italian market would have a capacity of *ca.* 3,000 tonnes/year. Thus, a process plant with dimensions described elsewhere [30] for the same annual production was taken into consideration to calculate that 228 m^3 of culture broth, containing 100 g/L of grown cells holding 77% of PHAs, could potentially be released into the target WWTP. An Italian WWTP for 20,000 person equivalents would have a 10,000 m^3 anoxic vessel with *ca.* 6 g/L of suspended sludge.[26,31] Therefore, assuming an instantaneous GMM point feeding (*i.e.* full discharge), the ratio between the suspended-biomass contained in the latter

vessel and the GMM-biomass entering is *ca.* 2.4 (60,000 kg of sludge/25,000 kg of GMM-biomass). Since the $ASSP_{Lab}$ had a 1 L-anoxic vessel holding *ca.* 5 g/L of suspended sludge, 2.1 g of GMM-biomass were fed. For this, 500 mL of *P. putida* KTOY06 culture grown to 4.2 g/L under optimal conditions (above) were centrifuged (8ºC, 6,797 rcf for 15 min) and the obtained pellet was suspended in 21 mL of mineral medium. It was concentrated to 100 g/L using a different culture medium (rather than LB medium) in order to obtain a more representative "industrial discharge" to be added into the anoxic chamber.

The $ASSP_{Lab}$ had been working for 5 days prior to the GMM-injection, allowing its performance to be verified (*e.g.* denitrification, COD reduction, etc.) under steady state conditions. Thereafter, all flows were temporarily stopped, the GMM-biomass was injected into the anoxic chamber, followed by stirring for 10 min to produce homogeneity of the exogenous cells within the indigenous sludge, and the pumps were restarted. To monitor the $ASSP_{Lab}$, 5 mL samples were withdrawn at -24, -2, 0 (injection time), 20', 40', 1, 1.75, 2.5, 4, 6, 11, 19, 24, 48 and 72 h from the anoxic and aerobic chambers and the settler sludge, while 50 mL were sampled from the settler supernatant and the accumulated-clarified effluent. After each sampling, the samples were divided into three parts: one portion was used immediately for cell counting (see below); a second was mixed with ethanol (50% v/v) and stored at -20 °C for DNA extraction and qPCR analysis (see below); and the rest was directly stored at -20 ºC until chemical analyses were performed (see below)

Modelling the fate of the GMM

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

- anaerobic vessel (Eq. 1):

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

- aerobic vessel (Eq. 2):

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

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

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

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

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

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

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

Analytical procedures

Quantitative PCR and colonies counting

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

target specifically the employed GMM through the Δ*fadBA* gene- were used for amplification. The primers were designed in this study, starting from previous descriptions [24] and were tested *in silico* against the *P. putida* KT2440 genome as negative set, resulting in no mismatches (as described in Supplementary Information Section S2.1). The qPCR protocol was previously optimized in order to have an efficiency ≥80% in the range of 10^2 to 10^7 copies and the lowest signal for the non-specific amplification of *P. putida* KT2440 used as negative control. The qPCR reactions (20 µL) contained 1x SsoAdvanced ™ Universal SYBR ® Green Supermix (Bio-Rad Laboratories S.r.l., Milan, Italy), forward and reverse primers 150 nM and 10 ng of DNA template. The temperature program for the 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 20 min. Specificity was checked with a final melt curve stage from 60 to 95°C at increments of 0.3°C. Linear 6-point calibrations in the range of 10^7 to 10^2 copies were obtained by using cloned *ΔfadBA* inserts as described in Supplementary Information Section S2.1; efficiencies ranged from 85 to 99%.

Plate counting was carried out using selective LB agar plates containing rifampicin and amphotericin B (after cooling) at final concentrations of 500 mg/L and 2.5 mg/L, respectively. Aseptic plating was performed using 30 µL of samples from serial dilutions. Colonies were counted after incubation at 30 °C overnight and concentrations calculated as colony forming units (CFU/mL).

Preliminary tests showed the qPCR and plate counting assays to be accurate quantification methods. This was validated by analysing different mixtures of *P. putida* KTOY06 and sludge in proportions from 1 (half sludge and half GMM) to 10^7 g_sludge/ g_GMM. Correspondence with expected values was verified: the qPCR was reliable in the range 1-10^5 (with errors <8%), while the signals obtained for higher ratios were at noise level (assigned to nonspecific amplification). The colony counting method

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

Monitoring the $ASSP_{Lab}$ performance

Soluble chemical oxygen demand (COD) was measured with a colorimetric kit (AQUALYTIC Vario MR), using glucose solutions for the calibration. Total nitrogen (TN) was measured spectrophotometrically as described in [32].

Ammonium ion concentration was measured by means of a selective electrode (CRISON 9663 C) and the results were expressed as ammonia nitrogen ($N\text{-}NH4^+$). Nitrate and phosphate were measured by ion-exchange chromatography (Dionex DX-120 with a column IonPac® AS14A) as described elsewhere [33]. Prior to analysis, samples were centrifuged (10,620 rcf, 10 min, 6°C), filtered (0.2 μm) and diluted as required.

Results and Discussion

$ASSP_{Lab}$ assessment

The $ASSP_{Lab}$ was functioning for 5 days before addition of the GMM-biomass, achieving steady-state conditions after 26 h from start-up. **Figure 2** shows the concentration profiles of target chemicals in all the $ASSP_{Lab}$'s vessels during the experimental period; points at -24 and -2 h were taken before GMM injection (0 h). The GMM addition caused perturbations in the concentration trends (TN, $N\text{-}NH_4$, $P\text{-}PO_4$ and COD). The $ASSP_{Lab}$ returned to steady state after 5 h (when variations of target chemical parameters were <10% of the respective mean values) and was monitored over the following 72 h. During the steady state, the $ASSP_{Lab}$ exhibited good performance in terms of TN, $P\text{-}PO_4$ and COD abatement; average falls in concentrations were (%): TN 48.4 ± 5.8, $N\text{-}NH_4^+$ 65.9 ± 6.2, $P\text{-}PO_4$ 16.6 ±

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

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

GMM monitoring and fate

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

The following initial conditions and rules were assessed: *i)* only the initial conditions for C_{Ar} *(0)*, C_{Cla} *(0)* and C_{Slu} *(0)* were fixed as zero value, while C_{An} *(0)* could be in the range of its average 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)* the GMM could settle or not, this determining the constraint $0.5 \leq i \leq 1$; *iii)* since the volume of the concentrated sludge in the settler was observed to vary between 0.45 and 0.5 L, the constraint $0.45 \leq i2 \leq 0.50$ was imposed. The resulting semilogarithmic concentration trends fit the experimental results very efficiently, with the following regression coefficients: R^2_{An} =0.81; R^2_{Ar} =0.99; $R^2_{\mathrm{Cla}} = 0.85$; $R^2_{\mathrm{Slu}} = 0.96$. All the resulting GMM generation rates were low absolute values (μ_{An} = -0.0832 h^{-1}, μ_{Ar} = -0.0383 h^{-1}, μ_{Cla} = 0.1117 h^{-1} *and* μ_{Slu} = 0.083 h^{-1}), as were those obtained for the NaCl injection, meaning that no significant GMM growth or death occurred in the $ASSP_{Lab}$. Only the GMM generation rate corresponding to the clarified zone was slightly higher than expected. This was considered a consequence of the sampling procedure for the settler-vessel since decanted-sludge was inevitably slightly resuspended when using the sampling pipette. The experimental and modelled results were transformed into totalities along the experimental time, namely: total GMM contained in the $ASSP_{Lab}$, quantity discharged by the clarified stream and amount eliminated by the concentrated sludge purge (**Figure 4**A). The same fitting curves are shown with the qPCR results in Figure 4B, where highly similar behaviours were detected. This implies that the number of mutant gene traits detected inside, and released by, the $ASSP_{Lab}$ was consistent with the number of CFU, thus indicating that GMM cells were mostly alive at the time of release and that no cell or DNA depletion occurred inside the $ASSP_{Lab}$. This is again concerning in terms of ecological impact, because the viability of this GMM strain has not been tested in environment-like conditions so far; a gradual input of a determinate species into an ecological niche increases the genetic frequency of particular gene traits, altering the gene pool, even if those traits have low fitness. In contrast to a report [23] for a different type of treatment plant (as mentioned above), the GMM reached the settler vessel 20 min after injection. Moreover, a significant

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

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

Effluent disinfection

The clarified stream is usually sent to tertiary treatment (*i.e.* free available chlorine (FAC), monochloramine (NH_2Cl), ozone (O_3), chlorine dioxide (ClO_2), and UV light-based strategies, used alone or in combination) to disinfect the water. Hence, this step becomes important to avoid the leakage of GMMs to the environment. This would not only deplete the viable GMM cells released from the WWTP, but would also notably lower the concentration of the mutant gene traits available for horizontal transfer [38]. Thus, further investigation should be carried out in order to determine the possible effects of the tertiary treatment on GMM containment. For example, it would be interesting to assess the depletion rate of the recombinant DNA at that stage to ascertain that, although GMMs might not be contained by the ASSP, the genetic material would hardly pass through it. Here, a preliminary hypochlorite-disinfection test was performed on the clarified $ASSP_{Lab}$ effluent in order to verify the abatement of living GMMs (CFUs). A portion of the collected clarified effluent was batch-treated with a commercial 4.5% NaOCl solution (2 mg/L final concentration) for 20 min, simulating continuous disinfection. The GMM cells, the initial concentration of which was $2.56x10^7 \pm 4.72x10^6$ CFU/mL,

were completely abated. The same test was carried out on the fully grown GMM culture; in this case the GMM concentration was reduced from $1.50x10^7 \pm 2.89x10^6$ to $6.67x10^1$ CFU/mL.

Risk assessment

The worst-case scenario of a pulse/spike GMM discharge was considered in order to assess the containment capability of a municipal ASSP in the event of an exceptional release. An actual industrial release would last hours instead of seconds (considering a discharge of the $228m^3$ fermenter hypothesised and described in the Experimental Approach section), and the GMMs would be diluted in the sewer-line with the pre-existing matrix (instead of directly entering the WWTP). This could result in a better mixing of GMMs with the whole WWTP-influent and sludge already in the ASSP, limited retention time variation in each ASSP-chambers and consequently better GMM-containment. On the other hand, to evaluate potential adaptation of the GMMs to the new environmental conditions, a worst-case scenario could also consider continuous discharge (deliberate or unintentional), or extending the experimental evaluation time to increase the probability of GMM adaptation to ASSP conditions. The GMM *P. putida* KTOY06 used in this study did not proliferate throughout the experiment. To extend the experimental time, the employment of actual wastewater (to feed the $ASSP_{Lab}$) is recommended to diminish/avoid effects on sludge features. Indeed, it is important to consider possible limitations of using a synthetic feeding solution – carrying neither naturally occurring microorganism nor suspended solids - during the experiment. This may influence two aspects, namely: *i)* the minimization of GMM-bacteria predation by protozoa; and *ii)* incorporation of GMM-cells in flocs. The former might overestimate the spiked GMM not contained by the $ASSP_{Lab}$. This fits with the above “worst case scenario”. For the second issue, the limited flocculation due to the lack of suspended solids in the feed is considered to have negligible impact on influencing GMM containment by the system, considering the flocs occurring in the actual sludge used to start-up the $ASSP_{Lab}$ (see Bench-scale activated sludge system and its start-up). Moreover, since the sedimentation efficiency did not

significantly change during the entire experiment (Figure 3), nor the sludge aspect for the first 48 hours, it could be inferred that feeding with synthetic wastewater did not alter significantly the original sludge characteristics and behaviour during the short period of the study. Moreover, after 24 h of injection - during which potential sludge alteration was even less - the GMM concentration in the $ASSP_{Lab}$ diminished to *ca.* 1/10 of the injected amount (Figure 4) by leaving the system through the clarified effluent or concentrated-sludge-purge streams (F_5 and F_7). Notwithstanding the potential experimental limitations, which could be more representative of a worst-case scenario, the results support the importance of risk assessment of GMM release outside biotech facilities.

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

Conclusion

A bench-scale activated sludge system ($ASSP_{Lab}$) was set-up to simulate an industrial discharge containing GMMs into a conventional municipal WWTP, to study its containment potential.
The results showed that if a PHA-producing GMM reached the ASSP of a small municipal WWTP (20,000 PE), they would be released into the environment through the clarified stream rather than being contained. Hence, tertiary treatment becomes particularly important in order to avoid such release.

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FIGURE and LEGENDS

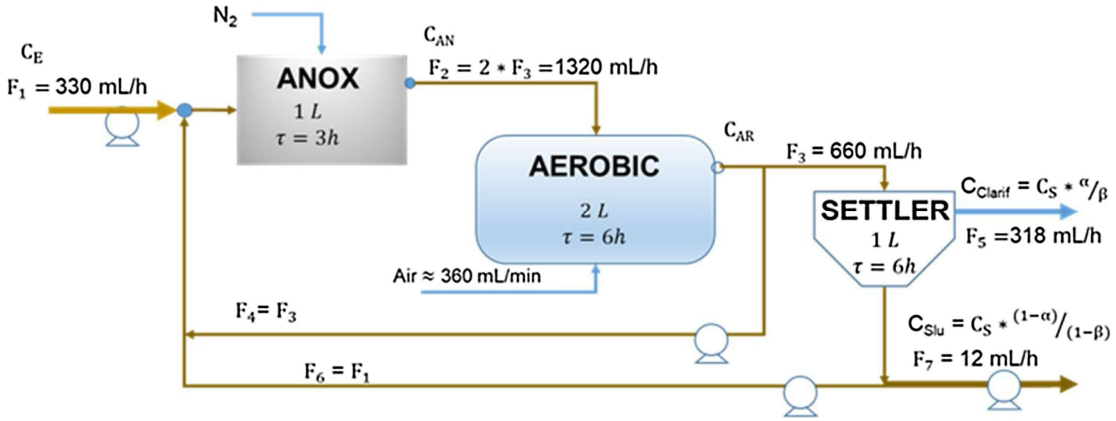


Figure 1: $ASSP_{Lab}$ configuration and set up. Flows (F_n) and GMM concentrations variables for the anoxic reactor (C_{AN}), aerobic reactor (C_{AR}) clarified effluent (C_{Clarif}) and concentrated sludge (C_{Slu}) are shown.

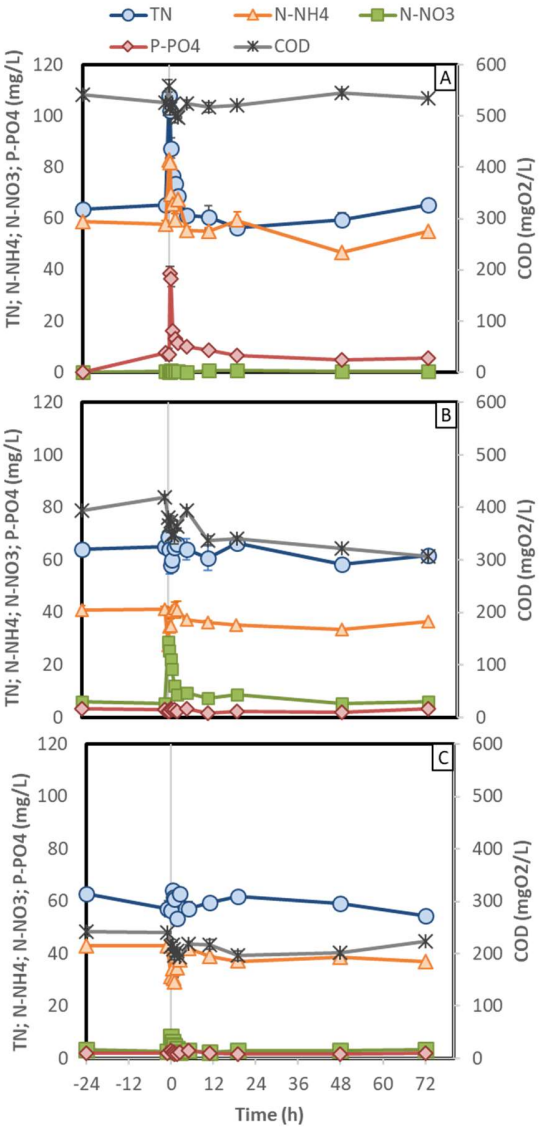
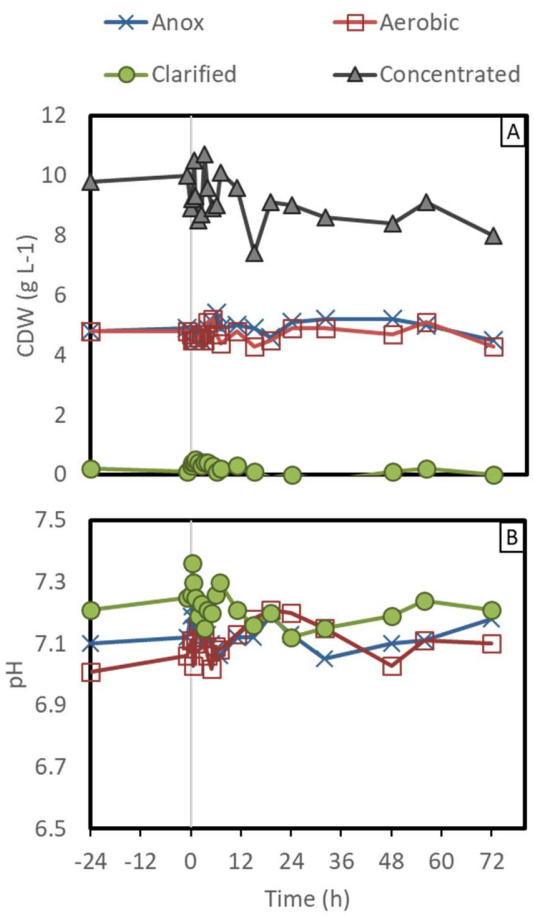


Figure 2: $ASSP_{Lab}$ performances. Concentration trends during the experiment in the anoxic (A) and aerobic (B) vessels, and in the clarified effluent (C).



1 **Figure 3:** $ASSP_{Lab}$ 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

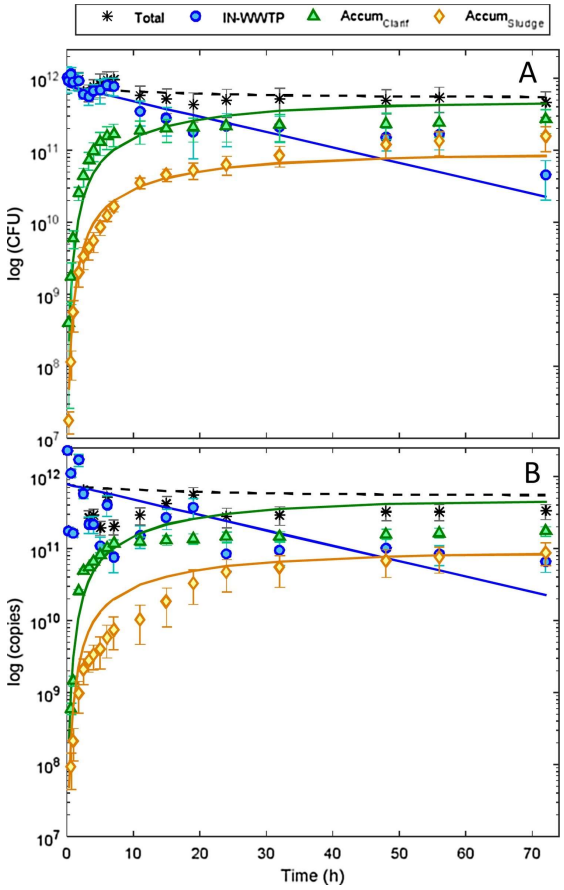


Figure 4: Fate of the GMM after being injected in the ASSP$_{Lab}$. Total amount of cells quantified along the experimental time by plate counting (A) and qPCR (B). Measurements allowed to quantify the GMM: inside the ASSP$_{Lab}$ (IN-WWTP), discharged through the clarified and purged streams (Accum$_{Clarif}$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