Tracing the evolution of degraders in activated sludge during the sludge’s acclimation to a xenobiotic organic

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Abstract. The molecular biology method of high-throughput pyrosequencing was employed to examine the change of activated sludge community structures during the process in which activated sludge was acclimated to and degraded a target xenobiotic. The sample xenobiotic organic compound used as the activated sludge acclimation target was the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Indigenous activated sludge microorganisms were acclimated to 2,4-D as the sole carbon source in both the batch and the continuous-flow reaction modes. Sludge masses at multiple time points during the course of acclimation were subjected to pyrosequencing targeting the microorganisms’ 16S rRNA genes. With the bacterial 16S rRNA sequencing results the genera that increased in abundance were checked with degradative pathway databases or literature to confirm that they are commonly seen as potent degraders of 2,4-D. From this systematic examination of degrader changes at time points during activated sludge acclimation and degradation of the target xenobiotic, the trend of degrader evolution in activated sludge over the sludge’s acclimation process to a xenobiotic was traced.

1. Introduction
Activated sludge microorganisms generally grow on substrates made of organic pollutants that are biogenic in nature. Under this natural situation, activated sludge microorganisms are not reactive with (indigenous to) any xenobiotic compound and are unable to degrade the xenobiotic. Fortunately, activated sludge can develop xenobiotic degradation capability after an acclimation process in which indigenous sludge is placed in reaction with the target xenobiotic for a sufficient period until degradation starts and proceeds to completion [1, 2]. Through the process of acclimation, some indigenous microorganisms, mainly bacteria, would acquire xenobiotic degradation capability and are converted degraders, while some would remain incapable (non-degraders) [2, 3]. How degraders evolve is an interesting and pressing question; determination of degrader evolution, in amount and its variation over time, serves the correct description of activated sludge’s degradative strength which dictates the behaviour and performance of the xenobiotic treatment sludge [4, 5].

It has long been recognized that the requirement of suitable methods for the identification and still more preferably, quantification of xenobiotic degrader be readily available. So far, there is seldom a claim for the most useful method to achieve such a requirement. The review of Widada et al. [6] has highlighted all previous molecular techniques for monitoring xenobiotic degrader bacteria, including the relatively recent methods of DGGE and rt-PCR. However, tracing degrader all through the process in which degradability develops is scantly reported, if any, in the literature. High-throughput pyrosequencing provides readings that can be translated to bacterial taxa to show microbial
community structures at any time point [7]. When specific genus can be examined, by off-line methods currently, for its potential xenobiotic degradability, these pyrosequencing readings may be used to identify degraders that are contained within microbial communities such as those in activated sludge. The purposes of this study were to analyze activated sludge community structure using high-throughput pyrosequencing, and with the feasibility of off-line degrader identification, to trace the trend and dynamics of degrader evolution during activated sludge acclimation and degradation of a xenobiotic. Trends of degrader evolution in the common acclimation reactions of batch and continuous-flow modes were determined which revealed the difference in amount of degrader obtainable due to different acclimation pressure exerted by the xenobiotic on the activated sludge biomass [3, 8]. In addition to devising a novel method that traces degrader evolution, this study also disclose the actual differences and changes in amount of degraders that happen in reactors that are commonly used in practical applications.

2. Materials and methods

2.1. Activated sludge and sample xenobiotic

The sample xenobiotic compound used as the activated sludge acclimation/degradation target was the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D).

The initial activated sludge seed was obtained from soil that did not have any record of 2,4-D nor metal contamination. The mixed culture from soil was grown to a suitable amount on Nutrient Broth (NB Difco 234000) for multiple (at least three) subcultures in multiple shake-flasks. The shake-flask microorganisms were considered indigenous and were used as seed to a separate fed-batch reactor for cultivation of sludge biomass.

2.2. Reactor operations

Fed-batch reactor cultivated and maintained indigenous activated sludge that was used at different times or batches that were to acclimate the biomass to 2,4-D. Acclimation reactions were performed in two (2) types of reactors: 1) Batch reactors for acclimation of indigenous activated sludge to 2,4-D; and 2) continuous flow reactors (CSTR) fed solely 2,4-D for acclimating indigenous activated sludge to 2,4-D that was input continuously. Shake-flasks were 1000 mL conical flasks, each contained a medium volume of 400 mL initially. CSTRs had built-in clarifier and a reactor volume of 3 L. Feeds to all reactors also contained minerals of FeCl₃, 1.2 mg/L; CaCl₂, 12 mg/L; MgSO₄.7H₂O, 65 mg/L; NH₄Cl, 125 mg/L; K₂HPO₄, 200.0 mg/L; and KH₂PO₄, 156.6 mg/L. These minerals are typically used in synthetic wastewater with which pH is buffered and essential nutrients are provided in quantities not limiting bacterial growth. Reactors used for acclimation activated sludge to 2,4-D were operated such that acclimation was completed, as shown from the non-detectable level of 2,4-D in the effluent. For both the batch and CSTR acclimation reactors, sludge was harvested from each reactor in regular interval (typically one day). The sludge masses were analyzed for their community structures by the process of high throughput pyrosequencing.

Measurement of activated sludge concentration was by suspended solids (SS) according to ‘Total Suspended Solids Dried at 103-105°C of Standard Methods [8]; measurement of 2,4-D was performed by HPLC [9].

2.3. Pyrosequencing

Activated sludge biomasses that were to acclimate to 2,4-D in batch and CSTR reactors were subjected to 16S rRNA gene pyrosequencing analyses. 16S rRNA gene is present in almost all bacteria and is currently used as a standard genetic marker for bacteria identification. Before pyrosequencing procedures the batch reactor sludge from multiple shake-flasks and the CSTR sludge from duplicate reactors were mixed in equal mass proportions. Equal masses of these sludge mixtures were subjected to pyrosequencing. Pyrosequencing was performed by a local biotechnology laboratory, using 454 GS Junior System following protocols recommended by the manufacturer.
2.4. **Degrader identification**

The abundances of all bacteria at the family level were first studied about their 2,4-D degradability. All bacteria present in the sludge, each at the genus level, were separately verified for their true 2,4-D degrader characteristics with references firstly to gene and pathway data aggregator websites, such as MetaCyc [10, 11], and when a particular species’ 2,4-D degradability was uncertain from the pathways databases, confirmation of such degradability was extensively searched from published literature.

3. **Results and discussion**

3.1. **Degrader identification**

Activated sludge biomasses, starting at their indigenous states, were acclimated and after which degraded 2,4-D in the batch and continuous flow reactors. From the high-throughput sequencing results, the relative abundances (% of all readings) of all bacteria under the taxa level of genus were recorded. Each genus was searched for its 2,4-D degradative characteristics from degradation pathway databases. Degraders within each of the sludge masses were identified and their relative abundances were determined. Simultaneously, non-degraders and their abundances were similarly determined.

Using the result of the CSTR acclimated sludge as an example, Figure 1 shows the courses of rise and fall of some prominent degraders, as well as those of some non-degraders. Genus names for the degraders and non-degraders are shown in the legend. The sum of the total number of degrader bacteria is shown as the curve indicated (and coloured red). In contrast, the black curve is the relative amount (number) of non-degraders. The degrader curve shows the evolution dynamics of degraders during activated sludge acclimation in the continuous flow reaction. Degrader and non-degrader followed a harmonious succession with each other.

![Figure 1. Total degrader summed up of individual genus that was verified 2,4-D degradative, while the rest were non-degraders. Legends show genera most prominent as degrader or non-degraders](image)

3.2. **Mapping degradation and degrader trends**

Figure 2a shows the growth of sludge (represented by SS) and the disappearance of 2,4-D during the batch acclimation process. In the batch reaction, acclimation of sludge to 2,4-D was achieved on about the third day (typical of most batch acclimation of activated sludge to 2,4-D), after which 2,4-D was degraded in a noticeably high rate. Figure 2b shows the evolution of degrader (and also the corresponding non-degrader changes) in the batch-acclimation reaction. Mapping the time of total...
degrader variation with that of 2,4-D degradation, it is shown that the degraders increased immediately at the start of acclimation reaction, and rose to a highest quantity on about the 4th day. When degradation of 2,4-D was complete (after Day-5), the number of degraders declined to a level that was slightly higher than that at the beginning of acclimation. At the last stage of 2,4-D degradation, sludge microorganisms are able to metabolize the downstream products for growth. With the depletion of 2,4-D and therefore diminishing of acclimation force, degrader abundance gradually gave way to non-degraders. At the continuous absence of the xenobiotic, degraders would revert to non-degraders (de-acclimation) gradually [5].

**Figure 2.** 2,4-D degrader evolution trends corresponding to 2,4-D degradation in batch acclimation and degradation reaction. (a) 2,4-D degradation (left axis) and SS growth (right axis) of duplicate tests; (b) degrader evolution (left axis).

**Figure 3.** 2,4-D degrader evolution trend corresponding to 2,4-D degradation in continuous-flow acclimation and degradation reaction. (a) 2,4-D degradation (left axis) and SS growth (right axis) of duplicate tests; (b) degrader evolution (left axis).
Figure 3a shows the growth of sludge and the disappearance of 2,4-D during the process of continuous flow acclimation. In a continuous flow reaction, influent 2,4-D, as a non-reactive compound, must reach a concentration theoretically equal to that of the influent after a hydraulic residence time. Only then the high 2,4-D concentration puts the sludge under substantial acclimation pressure. After this delay of 2,4-D concentration build-up, continuous flow acclimation was completed on about the 4th day. The rise of degraders in continuous-flow acclimation (Figure 3b) is similar to that of the batch acclimation with rapid increase when acclimation was the main activity. Also, degrader number reached the highest point when degradation of 2,4-D was complete (2,4-D effluent as low as non-detectable). After degradation was achieved, 2,4-D was still being continuously input so that mass of 2,4-D was continuously present in spite its concentration was significantly diluted in the reaction tank. The need for degrader bacteria kept degrader abundance at a certain high level. When the continuous flow reaction reached steady state, degrader content also reached a steady level that was a little lower than that of the maximum.

Figure 4 displays in parallel the evolutionary dynamics of degraders during the acclimation of activated sludge to a xenobiotic organics in batch and continuous flow reactors. In batch reaction, the starting concentration of 2,4-D is high to exert a high acclimation pressure to the sludge. The sum of degrading genera increased quickly during batch acclimation, in close concurrent to the degradation of the xenobiotic as a growth substrate. Approaching the end of acclimation where the target was depleted, degrader number decreased to certain level pending further reduction under de-acclimation. Acclimation in CSTR suffers a lag due to an initial dilution. Rise of degraders after the lag had a rate similar to batch acclimation, but the proportion of degrader was at all times lower than that attainable by batch acclimation. CSTR acclimation maintained a high degrader level (at approximately the maximum) and remained at that level for a length of time until the steady state when degrader was lowered. Compared to CSTR acclimation, batch acclimation yielded higher amount of degrader, but could maintain this amount for shorter period of time.

4. Conclusions
In this study, the evolution dynamics of xenobiotic degraders during the acclimation of activated sludge to the xenobiotic matter was obtained using an up-to-date molecular biological technology. Conclusions can be drawn:

- Xenobiotic degraders, due to the acclimation pressure, rise at a fast rate.
• The overall amount of degrader present in the reactor depends on the concentration of the acclimating xenobiotic. Degrader amounts decline rapidly at low xenobiotic concentration, but this is only more accurate after depletion of the xenobiotic than when xenobiotic is being diluted yet its mass is being continuously input.

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