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Monitoring microaerobic denitrification of *Pseudomonas aeruginosa* by online NAD(P)H fluorescence

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Abstract Defined as the transition conditions in which the organism(s) performs simultaneous aerobic and anaerobic respiration or fermentation, microaerobic conditions are commonly present in the nature. Microaerobic metabolism of microorganisms is however poorly characterized. Being extremely sensitive to the change in cellular electron-accepting mechanisms, NAD(P)H fluorescence provides a useful ways for online monitoring of microaerobic metabolism. Its application to studies of microbial nitrate respiration and particularly, denitrification of *Pseudomonas aeruginosa* is reviewed here, centering on four topics: (1) online monitoring of *anaerobic* nitrate respiration by NAD(P)H fluorescence, (2) effects of denitrification on *P. aeruginosa* phenotypes, (3) microaerobic denitrification of *P. aeruginosa* in continuous culture, and (4) correlation between NAD(P)H fluorescence and denitrification-to-respiration ratio. Online NAD(P)H fluorescence is shown to sensitively detect the changes of cellular metabolism. For example, it revealed the intermediate nitrite accumulation in C-limited *Escherichia coli* performing anaerobic nitrate respiration via dissimilative ammonification, by exhibiting two-stage profiles with intriguing fluorescence oscillation. When applied to continuous culture studies of *P. aeruginosa* (ATCC 9027), the online fluorescence helped to identify that the bacterium conducted denitrification even at DO > 1 mg/l. In addition, the fluorescence profile showed a unique correlation with the fraction of electrons accepted by denitrification (out of all the electrons accepted by aerobic and anaerobic respiration). The applicability of online NAD(P)H fluorescence in monitoring and quantitatively describing the sensitive microaerobic state of microorganisms is clearly demonstrated.

Keywords Microaerobic metabolism · Denitrification · Ammonification · *P. aeruginosa* phenotype

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Introduction

Oxygen is only scarcely soluble in water. Consequently, a continuous and dynamic spectrum of aerobic, microaerobic, and anaerobic or anoxic conditions occurs in the heterogeneous, complex environments, depending on the distributions of water flow, nutrients and microbial populations. It is known that the ecological fate of different organic compounds varies differently with the availability of oxygen [24]. Oxygenated compounds are largely biodegradable in all conditions, although the rates differ under aerobic versus anaerobic/anoxic conditions [21]. Highly chlorinated hydrocarbons, on the other hand, are more susceptible to sequential degradation: a reductive dechlorination under anoxic conditions (e.g., by sulfate-reducing bacteria or methanogens) followed by aerobic mineralization [34]. However, typical studies have been done under either fully aerobic or completely anaerobic/anoxic conditions. Despite its common existence in the nature, *microaerobic* conditions are not clearly defined. Microbial metabolism under the microaerobic conditions is even less well characterized.

From the viewpoint of microbial respiration, aerobic conditions correspond to those in which the organism(s) uses O_2 as the terminal electron acceptor (i.e., performs aerobic respiration); anaerobic or anoxic conditions correspond to those in which the organism(s) performs fermentation (without external terminal electron acceptors) or uses chemicals other than O_2 as terminal electron acceptors (i.e., performs anaerobic respiration) [24]. Accordingly, microaerobic conditions may be defined as the transition conditions in which the organism(s)

performs simultaneous aerobic and anaerobic respiration or fermentation.

Dissolved oxygen concentration (DO) provides a quantitative measure of the oxygen availability to the organisms. However, the same DO does not necessarily mean the same to different organisms or even to the same organism under different nutritional or environmental conditions. Techniques that allow direct assessment of the state of microbial respiration (aerobic vs. anaerobic) are therefore very useful to the study and characterization of the microaerobic environment. Such knowledge is essential for modeling the ecological fate of various substances for risk assessment and management as well as for the development of advanced bioremediation technology.

The ubiquitous *Pseudomonas aeruginosa* has versatile metabolic capability and is among the organisms most commonly isolated from petroleum-contaminated soils and groundwater [29]. Various strains of the bacterium are known to degrade aliphatic, aromatic, and polyaromatic hydrocarbons [2, 3]. *P. aeruginosa* is also an opportunistic pathogen causing pneumonia [7, 8], nosocomial bloodstream infections [9], and serious lung infection in the cystic fibrosis patients [26, 28]. The bacterium undergoes significant phenotype changes responding to varying DO in the environment. Being an active denitrifier [4, 5, 10, 17, 23], the bacterium has been reported to grow fast without mucoid under aerobic conditions, but turn mucoid and form biofilm matrix under microaerobic or anaerobic conditions in the presence of nitrate [11, 35]. Understanding the physiological responses of *P. aeruginosa* to denitrification at different DO is therefore of environmental, industrial, and medical significance.

Fluorescence of NAD(P)H, i.e., the reduced forms of the nicotinamide adenine dinucleotide coenzymes NAD and NADP (phosphorylated), offers a distinct potential for studying and monitoring the microaerobic activities of biological systems. Universally present in living cells, NAD(P) are the major intermediate electron and hydrogen carriers that couple the substrate catabolism with the respiration and anabolism [17, 33] (Fig. 1). Because only the reduced coenzymes NAD(P)H, not their oxidized counterparts $NAD(P)^+$, are fluorescent (excitation/emission maxima ~340/460 nm), the fluorescence intensity reflects the kinetic balance of NAD(P)H generation and consumption and is extremely sensitive to the change in cellular electron-accepting mechanisms [19, 33, 37]. In this work, the denitrification of *P. aeruginosa* as well as its online monitoring based on NAD(P)H fluorescence are reviewed.

Online monitoring of *anaerobic* nitrate respiration by NAD(P)H fluorescence

Many microorganisms can use electron acceptors other than oxygen for anaerobic respiration [24]. Common examples include nitrate (NO_3^-), sulfate (SO_4^{2-}), sulfur (S^0), carbonate (CO_3^{2-}), Fe^{3+}, Mn^{4+}, fumarate, etc. Among them, nitrate is the most common used by facultative aerobes. The *dissimilative* nitrate reduction (respiration) for energy generation is restricted to bacteria, although a wide variety of which can carry out this process. On the contrary, *assimilative* nitrate reduction, where nitrate is used as a nitrogen source for growth, occurs in all plants, most fungi, and many bacteria. In both dissimilative and assimilative reduction, nitrate is first converted to nitrite. Nitrite is then reduced to N_2 (dissimilative *denitrification*) or ammonia (assimilative or dissimilative *ammonification*). Accordingly, nitrate respiration proceeds via either denitrification (by denitrifiers such as *P. aeruginosa*) or dissimilative ammonification (most commonly by enteric bacteria such as *Escherichia coli*). Denitrification has been widely employed in biological wastewater treatment (WWT) processes [19, 27].

As indicated in Fig. 1, introduction of air to an originally anaerobic culture would increase the rate of NADH oxidation and decrease the NAD(P)H fluorescence. For bacteria capable of nitrate respiration, addition of nitrate would similarly cause a decrease in NAD(P)H fluorescence. The above phenomena have been clearly seen in studies with pure cultures of *P. aeruginosa*, *E. coli* and *Zymomonas mobilis*, and with WWT activated sludge [17, 19, 33]. The fluorescence drops affected by the nitrate addition ($\Delta_{NR} = NFU_{AN} - NFU_{NR}$) were found smaller than that by aeration ($\Delta_{OX} = NFU_{AN} - NFU_{OX}$), where NFU_{AN}, NFU_{NR}, and NFU_{OX} are the fluorescence intensities

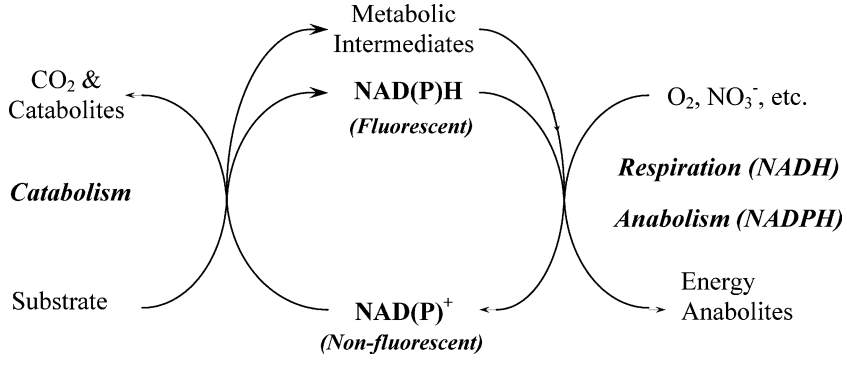

Fig. 1 The cyclic nature of coenzymes NADP and NADPH in heterotrophic metabolism

(in factory-calibrated normalized fluorescence unit (NFU)) under anaerobic, (anoxic) nitrate-reducing, and oxic (O_2-respiring) conditions, respectively. The ratios of Δ_{NR}/Δ_{OX} were 0.65–0.85 for *P. aeruginosa*, 0.70 ± 0.14 for *E. coli*, 0 for *Z. mobilis* (incapable of nitrate respiration), and 0.7 ± 0.1 for the activate sludge from a WWT plant (Oaks, PA, USA) employing a biological nutrient removal (BNR) process [17, 19, 33]. The finding of smaller Δ_{NR} than Δ_{OX} was attributed to the slower NADH oxidation associated with nitrate reduction than that with aerobic respiration, because nitrate is a weaker oxidant (with reduction potentials of +0.74 V $[NO_3^-/N_2]$ and +0.70 V $[NO_3^-/NH_3]$ for denitrification and dissimilative ammonification, respectively) than O_2 (with a reduction potential of +0.82 V for respiration).

The effectiveness of online monitoring of nitrate respiration based on NAD(P)H fluorescence was particularly demonstrated in the study with resting cultures of *E. coli* (Fig. 2) [18, 33]. *E. coli* responded to nitrate addition with either one-stage (Fig. 2a) or two-stage (Fig. 2b) profiles. (In the studies conducted so far, *P. aeruginosa* had been observed to always exhibit the one-stage response.) The one-stage response corresponded to the case of no transient accumulation of intermediates during the nitrate respiration. The observed two-stage response of *E. coli* was confirmed to result from intermediate nitrite accumulation. When nitrate became depleted, the fluorescence increased to a mid-level at the end of the first stage. The nitrite accumulated in the first stage was then slowly consumed (reduced) by the bacterium during the second stage. Because the study was conducted under C substrate-limiting condition, it appeared that the nitrite reductases of *E. coli* were less stable than the nitrate reductases.

Furthermore, an intriguingly oscillation of the fluorescence was observed with the cultures showing the two-stage responses (Fig. 2b), but was never seen with the cultures showing the one-stage responses. The persistent fluorescence oscillation was particularly clear during the second stage. The exact origin of the oscillation remained unknown [18]. Nonetheless, the resultant profiles clearly demonstrated the sensitivity of online NAD(P)H fluorescence to changes in cellular metabolism.

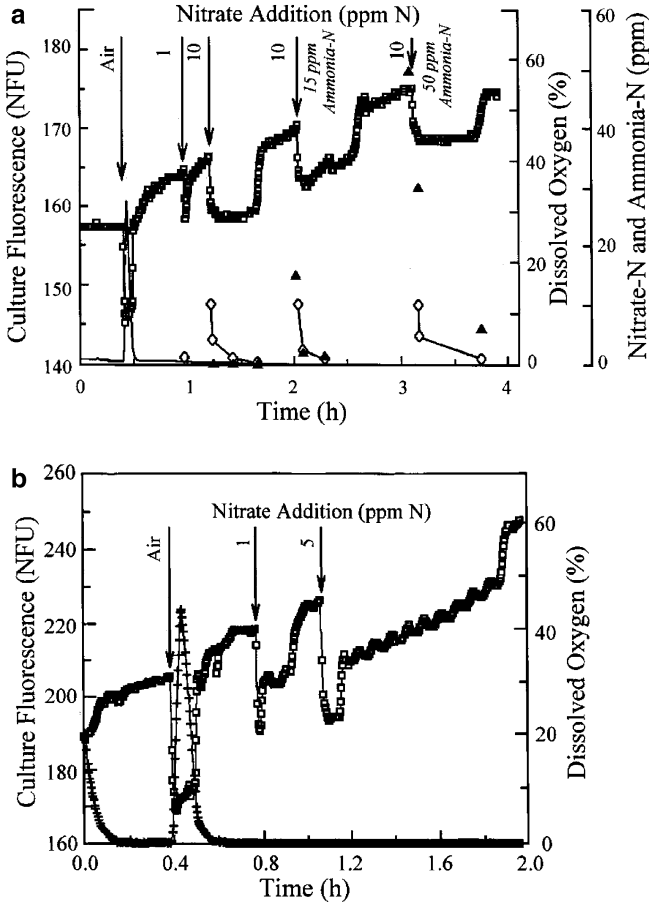

Fig. 2 NAD(P)H fluorescence profiles of *E. coli* showing **a** single-stage and **b** two-stage responses to nitrate addition; *open box* fluorescence; *lines* DO; *open diamond* NO_3N; and *open triangle* NH_4^+-N

Effects of denitrification on *P. aeruginosa* phenotypes

The Gram-negative bacterium *P. aeruginosa* exhibits two phenotypes: mucoid and nonmucoid. Typically occurring in the environment, the nonmucoid phenotype has high motility (with flagella or Type IV pili) and secretes high levels of proteases and siderophores [21]. The mucoid phenotype, on the other hand, is immotile and produces large amounts of alginate, exopolysaccharides and other virulence factors such as exotoxin A, exoenzyme, elastase, alkaline protease, phospholipase and rhamnolipids [30].

All nonmucoid wild-type *P. aeruginosa* strains have the genetic potential for spontaneous conversion to alginate-producing mucoid variants [11]. Although the genetic mechanisms are not fully defined, the conversion is thought to involve mutations in *muc*A, which encodes a cytoplasmic membrane-bound anti-sigma factor, MucA. When MucA is absent or incapable of binding its cognate sigma factor AlgT(U), active transcription of the alginate genes under its control occurs and the mucoid phenotype is expressed [25]. Physiologically, the conversion between the two phenotypes is triggered by the mechanism whereby the bacterium sensed the gradient of oxygen tension [13], as clearly indicated in Table 1 for the phenotypes established under different culture conditions [22]. The nonmucoid phenotype prevails when the bacterium senses the existence of an oxygen gradient (as in the static culture under surface aeration [Table 1]) and its flagellum-dependent motility enables the bacterium to move towards increasing oxygen tension. Without an oxygen gradient (as in the shaken culture with surface aeration), the bacterium exhibits the mucoid phenotype. Furthermore, the oxygen gradient-sensing mechanism of the organism appears to be paralyzed when using nitrate and/or nitrite

Table 1 *P. aeruginosa* phenotypes exhibited under different growth conditions

Growth condition	Phenotype
Static + surface aeration	Nonmucoid
Shaking + surface aeration	Mucoid
Shaking + nitrate (nitrite) + surface aeration	Mucoid
Static + surface aeration + nitrate (nitrite)	Mucoid
Anaerobic + nitrate (nitrite)	Mucoid

as the final electron acceptors. Even in the presence of oxygen gradient, the addition of nitrate would induce the nonmucoid to mucoid conversion [22]. Denitrification thus plays a very important role in the phenotype conversion of *P. aeruginosa*.

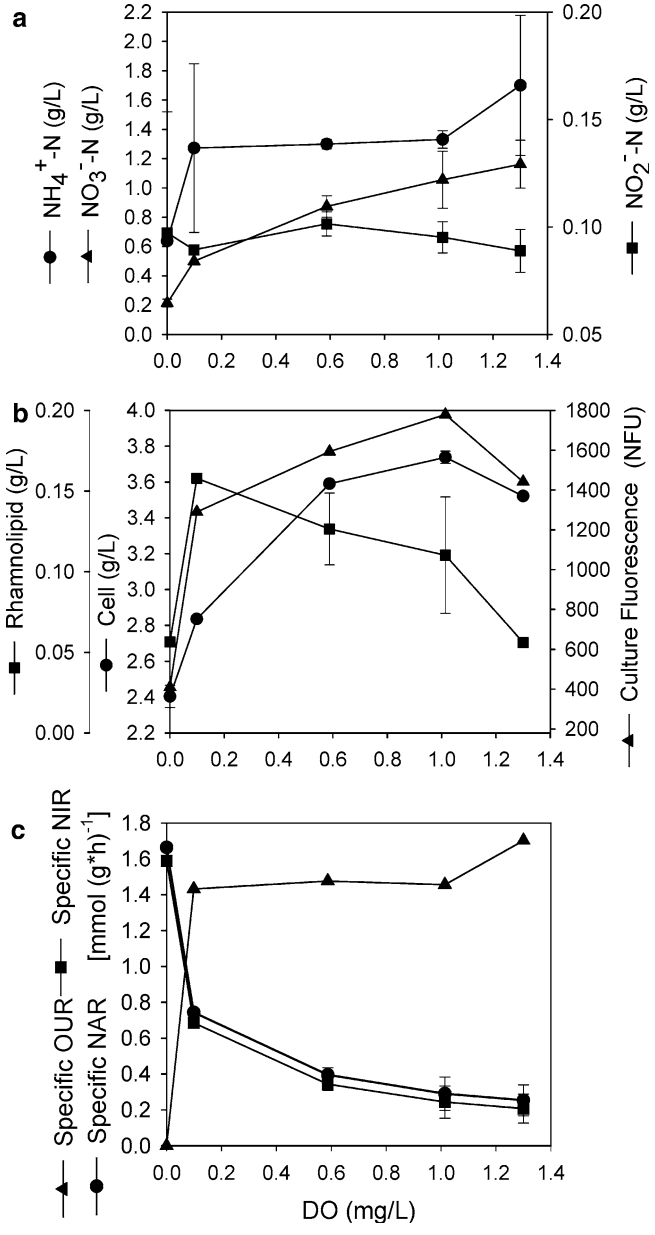

Fig. 3 Steady-state properties of *P. aeruginosa* in continuous cultures at D = 0.026 h^{-1} and different DO

The denitrification-affected phenotype conversion has significant effects on the biofilm formation and the pathogenicity of *P. aeruginosa*. The bacterium is an opportunistic pathogen that frequently causes pneumonia [7, 8], nosocomial bloodstream infections [9], and serious lung infection in the cystic fibrosis patients [26, 28]. In cystic fibrosis airway infection, *P. aeruginosa* undergoes the phenotype change from nonmucoid to mucoid [11, 14]. The secreted alginate exopolysaccharides help bacteria adhere to the host cells, form the biofilm of microcolony matrix, evade the host immune system response [26], and protect against phagocytosis, antibodies and antibiotic treatment [1, 11, 16, 32]. In a study of biofilm formation of *P. aeruginosa*, the anaerobic, denitrifying biofilm was found three-fold thicker and 1.8 fold higher in cell viability than the aerobic biofilm [36]. Several studies [13, 36] have also indicated the relationship between denitrification and the quorum-sensing systems of *P. aeruginosa* (which itself is a complicated topic beyond the scope of this current review). The capability of online monitoring the bacterial electron-accepting state (O_2 respiration vs. denitrification) will prove very beneficial in studying the complex metabolism of *P. aeruginosa*.

Aerobic denitrification of *P. aeruginosa* in continuous culture

In general, the enzymes involved in dissimilative nitrate reduction are repressed by O_2 and are synthesized under anaerobic/anoxic conditions [24]. In a continuous culture study with *P. aeruginosa* ATCC 9027 on glucose, the strain however performed denitrification even at DO > 1 mg/l [5]. For example, the steady-state culture properties measured at different DO for a dilution rate (D) of 0.026 h^{-1} are summarized in Fig. 3 [5]. At such a low-dilution rate, the concentrations of glucose (limiting nutrient) were practically zero in the broth and were not reported in the figure. With increasing DO, the specific NAR (nitrate reduction rate) decreased, consistent with the well-known repression and inhibition effects of oxygen [15]. The specific NIR (nitrite reduction rate) were only slightly smaller than the specific NAR, indicating that the nitrite formed from nitrate reduction was immediately converted through the subsequent denitrification pathway. Denitrification, however, persisted (at a specific NAR of 0.2–0.3 mmol/g/h) even at relatively high DO (1.0–1.3 mg/l). Although rare, similar phenomena, termed aerobic denitrification, have been observed with several microbial species and strains [20, 23], including *P. aeruginosa* [20, 38].

As shown in Fig. 3c, the specific OUR (oxygen uptake rate) approached the maximal level (~1.4 mmol O_2/g dry cells/h) even at the lowest nonzero DO (0.1 mg/l) studied [5]. For this *P. aeruginosa* strain, the Monod half-rate saturation constant for DO (i.e., the critical DO at which the specific OUR is half of the maximum rate) is apparently lower than 0.1 mg/l in the presence of

nitrate. For bioenergetics of *P. aeruginosa*, the known pathways for electron acceptance and ATP generation (from respiratory chain) are shown in Fig. 4 [31]. Accordingly, the specific ATP formation rate in the respiratory chain (FR_{ATP}, in mmol ATP/g dry cells/h) could be calculated: FR_{ATP} = (NAR + 3 NIR + 6 OUR)/x, where x is the cell concentration (g/l). The FR_{ATP} at different dilution rates (D) and DO are summarized in Fig. 5. FR_{ATP} was relatively constant for a given D, regardless of the different ratios of aerobic and anaerobic respiration employed in the oxidative phosphorylation. The observation implied that the aerobic denitrification functioned more as a supplementary energy-generating mechanism than as a competitive electron-accepting mechanism. As shown in Fig. 6, the average FR_{ATP} (for each D) increased linearly with increasing D, with the following best-fit equation: FR_{ATP} = (277.8) D + 4.29. The proportionality is reasonable considering the higher energy requirement for faster growth at a higher D. The intercept 4.29 (mmol of ATP per g of dry cells per h) corresponds likely to the required maintenance energy of the culture.

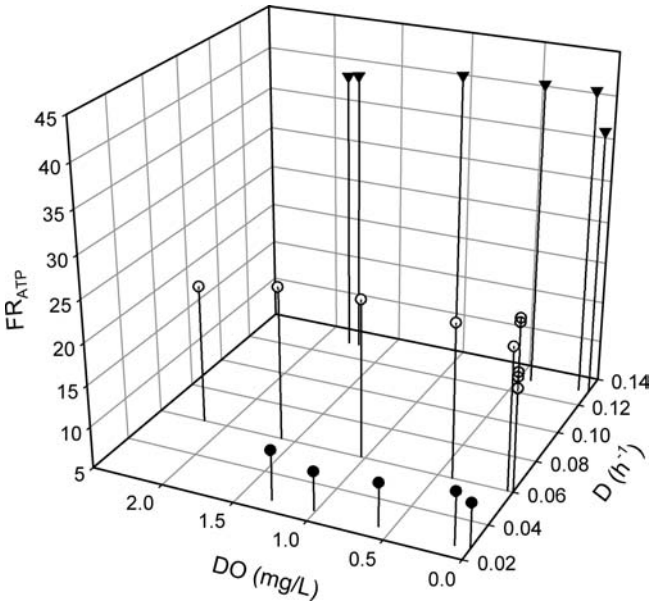

Fig. 5 Estimated specific rate of ATP formation via oxidative phosphorylation (FR_{ATP}) in continuous cultures of *P. aeruginosa* at various dilution rates (D) and DO. The electrons from glucose oxidation were accepted via O_2 respiration and denitrification in different ratios

Correlation between NAD(P)H fluorescence and denitrification-to-respiration ratio

As explained earlier and shown in Fig. 2, the fluorescence level would be higher under the fully anoxic, denitrifying conditions (NFU_{DN}) than under fully oxic (aerobic) conditions (NFU_{OX}). Under *microaerobic* conditions, NADH was oxidized partly by nitrate and nitrite and partly by oxygen. The resultant fluorescence (NFU) should therefore be at a level between NFU_{DN} and NFU_{OX}. In addition, the normalized fraction (NFU – NFU_{OX})/(NFU_{DN} – NFU_{OX}), ranging from "0" to "1", represents a quantitative indicator of the culture's "fractional approach" to the completely denitrifying state.

According to the electron-accepting pathways shown in Fig. 4, another quantitative indicator for the culture's extent of denitrification can be calculated, i.e., the fraction of electrons accepted by denitrification (out of all the electrons accepted by both aerobic respiration and denitrification): (2 NAR + 3 NIR)/(4 OUR + 2 NAR

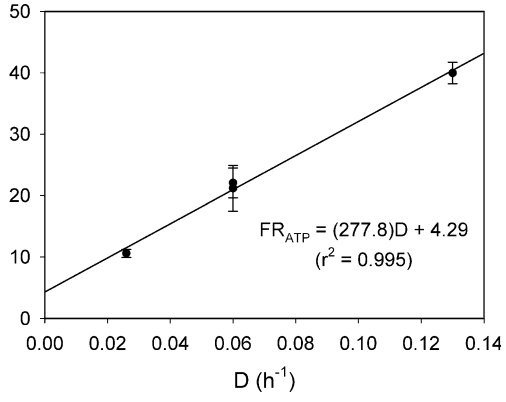

Fig. 6 Linear correlation of FR_{ATP} with D. The FR_{ATP} at each D were averaged from systems maintained at various DO at the same D

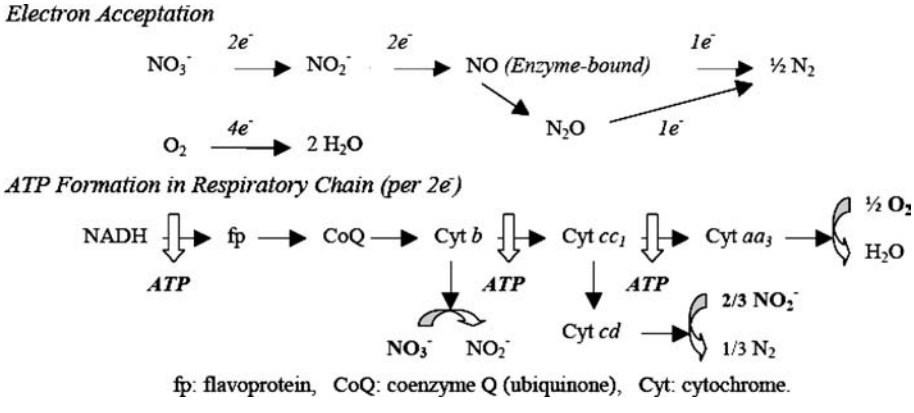

Fig. 4 Electron acceptance and ATP formation in respiratory chain of *P. aeruginosa*

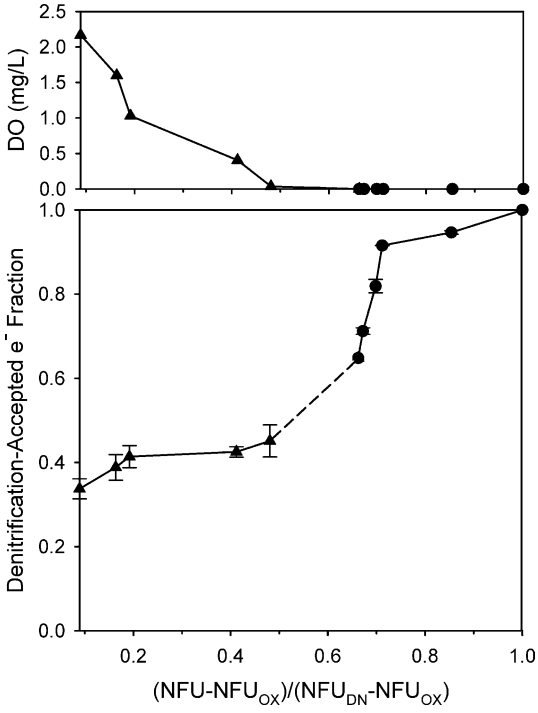

Fig. 7 Correlation between denitrification-accepted fraction of electrons and NAD(P)H fluorescence fraction for continuous cultures at D = 0.06 h^{-1} under various DO and aeration conditions. (Results were from two sets of experiments, with the presumed connection drawn as the *dashed line*, using feed media with two different glucose concentrations)

+ 3 NIR). Both the NFU fraction and the electron acceptance fraction have values between "0" and "1", with "0" corresponding to fully aerobic metabolism and "1" to fully (anaerobic) denitrifying metabolism.

Potential correlation between the two fractions has been attempted [5, 6]. For example, the results shown in Fig. 7 are for the continuous cultures at D = 0.06 h^{-1}. For demonstration of the sensitivity of the online fluorescence monitoring, some of the results in Fig. 7 were obtained from several zero-DO systems that differed only in the aeration rates employed [6]. As expected, the fraction of electrons accepted by denitrification increased with an increase in the NFU fraction. However, the data did not fall on the "ideal" diagonal, which would correspond to a directly proportional relationship. Instead, for the systems with nonzero DO, the profile was rather flat, indicating the less sensitive changes in the e^-accepting fraction than in the fluorescence fraction. The most sensitive changes in the denitrification-accepted e^- fraction occurred at the fluorescence fractions of $\sim$ 0.6 – 0.72. The fundamental significance of the unique shape of the correlation between the two fractions, reproducible trend wise in studies conducted at two other dilutions rates (D = 0.026 and 0.13 h^{-1}) [5, 6], remains to be further elucidated. Nevertheless, the results demonstrated the applicability of the online NAD(P)H fluorescence in monitoring and quantitatively describing the sensitive microaerobic state of microorganisms. Future study to gather data at different conditions and with various species and strains is warranted for establishing a clearer understanding of the relationship between the two fractions.

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