ENVIRONMENTAL SCIENCES 1999 Vol. 3, No. 3, 169~175
Bulletin of the Korean Environmental Sciences Society

Role of Sodium Ion in Biodegradation of Nitroaromatic Compound by Activated Sludge and Pure Cultures

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(Manuscript received on August 6, 1999)

2,4-Dinitrophenol(DNP) is a metabolic uncoupler that prevents cells from creating energy for growth and it has been suggested that the availability of sodium ions may be important in mitigating the effects of uncouplers. Accordingly, the degradation of DNP was investigated using activated sludge which had been adapted to mineralize DNP. After the acclimation of the activated sludge, the effect of sodium ions on the toxicity of high concentrations(80 to 100 mg/L) of DNP was investigated over a sodium ion concentration range of $9.3x10^{-5}$ to 94 mM. The concentration of sodium ions in the activated sludge mixed liquor seemed to have little effect on the DNP toxicity. However, a lack of sodium in the growth media resulted in a reduction of the DNP degradation rate by a bacterial isolate from the activated sludge culture identified as *Nocardia asteroides*.

Key words : Sequencing Batch Reactor(SBR), DNP, Sodium, Inhibition, *Nocardia asteroides.*, Activated Sludge

1. Introduction

The xenobiotic, 2,4-dinitrophenol(DNP), is commonly used in the production of dyes and explosives. Other forms of DNP such as 2,5- and 2, 6-dinitrophenols are used in the preservation of timber and as analytical indicators. Because of their widespread use and moderate solubility(195 mg/L @25℃), dinitrophenols now contaminate many natural waters. DNP is a toxic compound which remains persistently in the environment. Its resistance to biodegradation is thought to be a result of two factors : the presence of nitro-groups in the phenolic parent compound which deter any enzyme attacks[1] and, at higher concentrations, its behavior as an uncoupler of respiration and oxidative phosphorylation in cells(Fig. 1). As a result, while substrate degradation and the ETC(electron transport chain) transport of electrons continues, the synthesis of ATP and energy production ceases. Exposure to uncouplers such as DNP initially increases the oxygen uptake, yet eventually inhibits growth and reduces the cell mass yield[2,3].

Researchers[4,5,6,7] have proposed that one mechanism for overcoming the uncoupler interruption of the proton gradient and ATP phosphorylation is a switch to a transmembrane sodium ion gradient (Fig. 2). A sodium ion is pumped through the membrane during the passage of the electrons down the ETC. This exergonic transport of a sodium ion from outside to inside the membrane by an Na^+-ATP synthase provides energy for the synthesis of ATP. In respiring bacteria, the free energy from electron transport is coupled to a membrane proton pump that establishes a proton gradient which in turn drives the ATP phosphorylation using an ATP synthase. Recent evidence indicates that in some bacteria, a sodium ion can replace a proton as a coupling cation in these bioenergetic processes. Avetisyan et al.[4] found that an Na^+-motive driven respiration can be induced in *E. coli* grown either in the presence of a protonophore or at a high pH which interrupts the transmembrane proton gradient. Within the framework of the Na^+ cycle concept[5], it seems reasonable to assume that the Na^+-gradient is coupled to a specialized Na^+-ATP synthase enzyme to form ATP under conditions where the maintenance of the normal proton gra-

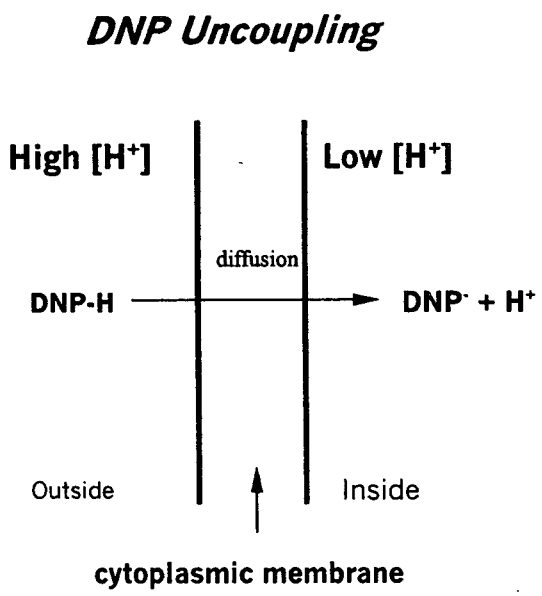

Fig. 1. The protonated DNP diffuses across the cytoplasmic membrane thereby discharging the electrochemical gradient generated by the electron transport chain.

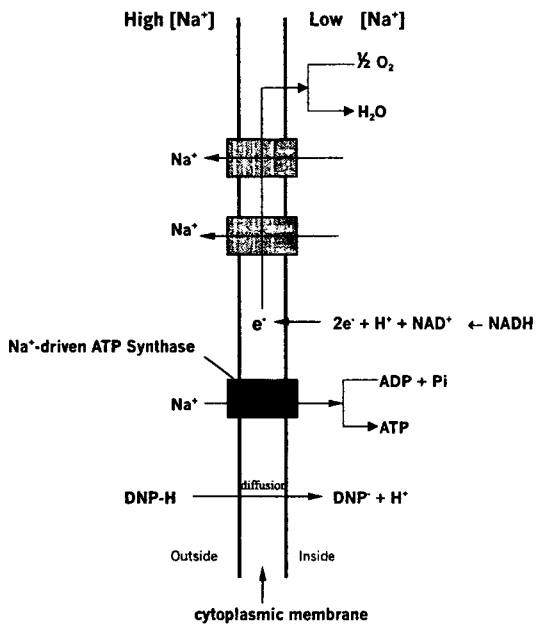

Fig. 2. Proposed model for uncoupling the electron transport and ATP synthesis through the generation of a sodium ion electrochemical gradient across the cytoplasmic membrane.

dient is difficult. Na^+-coupled nonoxidative phosphorylation has been reported in certain strains of anaerobic bacteria where an Na^+-gradient-coupled decarboxylase is used to produce ATP[6,7].

Previous research has revealed a variety of microorganisms that can degrade mononitrophenols in pure and mixed cultures[8,9,10,11]. A lot of early research was devoted to studying soil microorganisms, including *Arthrobacter* sp.[8,12] and strains of *Pseudomonas, Flavobacterium* and *Nocardia* spp.[13,14] that utilize *o*-nitrophenol(ONP) and *p*-nitrophenol(PNP) as microbial nutrients. While the microbial degradation of mononitrophenols has received much attention, there have been fewer studies on the degradation of dinitrophenol by pure cultures of microorganisms[1,8,9]. Gundersen and Jensen[8] isolated a bacterium, identified as *Corynebacterium simplex*, that degrades 2,4-DNP along with an approximately 50% stoichiometric accumulation of nitrite. Jensen and Lautrup-Larsen[9] also identified an *Arthrobacter* sp. and *Pseudomonas* sp. that can metabolize 2,4-DNP.

The objective of this study was to evaluate the effect of the environmental factor of an Na^+ concentration on enhancing the biodegradation of DNP by an acclimated activated sludge culture.

2. Materials and Methods

The reliance on sodium ions for mitigating DNP toxicity was investigated using activated sludge bacteria. A range of sodium ion concentrations was selected to bracket the tolerance range suggested for activated sludge cultures, $0 < [Na^+] < 93$ mM. Inhibition was detected as either a lag, a reduction in rate, or a complete loss of DNP degradation activity by acclimated activated sludge. The effect of sodium ion concentrations on DNP biodegradation with activated sludge cultures was investigated with DNP concentrations ranging between 80 and 100 mg/L. Other investigators have observed that bacteria can mineralize DNP in concentrations as high as 92 mg/L[15], however, inhibition also occurs more quickly at higher DNP concentrations, even with acclimated bacteria.

Bench-scale experiments were conducted using Sequencing Batch Reactors(SBR) with a 4-liter working volume including acclimated activated sludge for degrading the DNP, glucose as the

carbon/energy substrate, and KNO_3 as the nitrogen source for low DNP concentrations. This SBR activated sludge culture was then used as the inoculum for flask experiments on the effect of sodium ion concentrations on the degradation of relatively high concentrations of DNP.

2.1. Flask experiments

Short-term experiments were used to measure the DNP degradation by the SBR-acclimated activated sludge with different sodium ion concentrations. Each 250-mL flask contained 200 mL of the activated sludge mixed liquor along with a selected initial concentration of DNP(either 80 or 100 mg/L) and inorganic salts for nutrients. Accordingly, the flask biomass concentrations increased from 1,300 to 1,600 mg/L MLSS. Table 1 is a summary of the flask experimental conditions. The inorganic salts were added to the activated sludge inoculum to insure the balanced growth of the activated sludge bacteria. The flask liquid contents were aerated and mixed using a Gyrotory, Model G2 shaker(New Brunswick Scientific Co., Edison, NJ, USA) operating at 200 revolutions per minute, and allowed to react at room temperature, 22±2 ℃.

Table 1. Activated sludge flask reactor conditions for sodium ion effect experiments

Variable	Concentration (or as otherwise stated)
DNP (mg/L)	80 ~ 100
MLSS (mg/L)	1,306 ~ 1,630
C:N ratio (g/g)	6
pH	7.1 ± 0.1
Reaction period (hr)	24 ~ 36

The effect of sodium ions on inhibiting the activated sludge degradation of DNP was investigated over a sodium ion concentration range of $9.3x10^{-5}$ to 94 mM. During the tests, the sodium ion concentrations in the mixed liquor were maintained by the addition of sodium chloride. The sodium ion concentration in the growth media for the DNP-degrading activated sludge inoculum was 9.3 mM. The sodium ions in the activated sludge inoculum were removed by washing in a phosphate buffer. In the first set of experiments, the activated sludge cultures were washed once to achieve lower sodium ion concentrations of 0.93 mM. In the second set of tests, the activated sludge inoculum was washed six times in a phosphate buffer to reduce the sodium ion concentration in the mixed liquor to $9.3x10^{-5}$ mM.

Two initial concentrations of DNP were used with both the single-washed and six times-washed activated sludge cultures : 80 mg/L and 100 mg/L. These two concentration levels were assumed to be close to the level where DNP is inhibitory even to acclimated bacteria, therefore, the effect of sodium ions on mitigating the uncoupling effect of DNP was expected to be more visible.

2.2. Isolation of DNP-degrading bacteria from activated sludge

The SBRs were initially inoculated with cultures of both the actinomycete and the *Janthinobacterium* sp. to form an activated sludge biomass. This consortia of the two strains was then mixed with activated sludge obtained from a pharmaceutical company's waste treatment process. The *Janthinobacterium* sp. was apparently washed out of the sludge, therefore, the DNP degradation activity was produced by the other microorganisms. The SBRs had been operated for 4-years prior to this study. To examine the effect of sodium ions more closely, a bacteria strain was isolated from the activated sludge acclimated to 80 mg/L DNP. It was established that the isolate used DNP as the sole carbon and energy substrate and nitrogen source through extracting bacteria from the activated sludge flow using a method developed by Silverstein et al.[16] Spread plates were then made on a DNP-agar to grow colonies of DNP-degrading bacteria. Individual colonies were then successively replated on a noble agar containing 50 mg of DNP as the sole carbon and energy substrate along with inorganic salts, as described in Table 2. Subsequently, cells from purified colonies growing on single plates were inoculated into 250-mL Erlenmeyer flasks to test for DNP degradation in the broth. Finally, the DNP-agar plates were reinoculated with cells from the broth to check whether the pure culture had been contaminated. A cell wall lipid analysis was performed to identify the isolates using a com-

mercial database(Microcheck Inc., Northfield, VT, USA).

2.3. Pure culture experiment

The *Nocardia asteroides* isolate did not require any exogenously supplied growth factors and was grown in a liquid medium containing reagent grade DNP(Fluka Chemical Co., Ronkonkoma, NY, USA) as a source of carbon and energy along with the inorganic salts, as described in Table 2, except that dibasic sodium phosphate(Na_2HPO_4) was replaced with dibasic potassium phosphate($K_2H\ PO_4$) at a concentration of 0.81 g per liter. This procedure eliminated any sodium ions from the sterile growth media, apart from when added for the experimental variation of the sodium ion concentration. The effect of sodium ions on inhibiting the pure culture degradation of DNP was examined over a range of 0 to 93.3 mM. The sodium ion concentration was maintained during the tests by the addition of sodium chloride. The pH of all the growth media was maintained at neutral by a phosphate buffer, and no change in pH occurred as a result of DNP mineralization. It was anticipated that the pure cultures would be more sensitive to DNP than the organisms in the flocculant activated sludge biomass. Furthermore, the number of cells that could be maintained in the pure culture grown only on DNP was much smaller than the DNP-degrading biomass in the activated sludge. To avoid significant inhibition and measure the DNP degradation profiles within a reasonable reaction period, the initial concentration of DNP for the pure culture experiments was only 12 mg/L. The sodium ion concentration experiments with the bacterial isolate were conducted aerobically in 250-mL glass-stoppered Erlenmeyer flasks containing 200 mL of a liquid medium. The flasks contents were mixed using a Gyrotory, Model G2 shaker(New Brunswick Scientific Co., Edison, NJ, USA) operating at 200 revolutions per minute and allowed to react at room temperature, 22±2 ℃.

2.4. Analyses

Samples were taken from the flask reactors every 6 hours by syringe using a 16-cm^3 syringe fitted with a 20-gauge stainless steel needle. The samples were immediately vacuum filtered through a 0.2 μm polycarbonate filter(Nucleopore Corp., Pleasanton, CA, USA) and stored at 4 ℃ to prevent any bacterial activity.

Table 2. Bacterial growth media used for pure culture and activated sludge flask experiments on effect of sodium ions

Constituent	Activated sludge	*Nocardia*
	Concentration [mg/L] (or as otherwise stated)	
DNP	80 ~ 100	12
K_2HPO_4	810	810
KH_2PO_4	500	500
$MgSO_4 \cdot 7H_2O$	10	10
$CaCl_2$	10	10
NaCl	$9.3x10^{-5}$~107 mM	0~93.3 mM

The DNP content of the samples from both the activated sludge and the isolate containing the flask-mixed contents was measured by UV light absorbance at 260 nm using a spectrophotometer (Model UV 160V, Shimadzu Co., Kyoto, Japan). The biomass density was measured as mixed liquor suspended solids(MLSS) using the membrane filter technique outlined in Standard Methods[17].

The cell concentrations in the flasks containing the pure culture isolates were determined by the filtration of a 20-mL sample aliquot through an 0.2 μm polycarbonate filter(Nucleopore Corp., Pleasanton, CA, USA). The filters with the bacterial cells were then dried at 80 ℃ for 20 hours before weighing on an analytical balance.

3. Results and Discussion

The effect of the sodium ion concentrations on the DNP degradation with the single-washed activated sludge cultures is presented in Fig. 3 and 4. Fig. 3 shows profiles of DNP degradation in the presence of sodium ion concentrations from 0.93 to 93.93 mM ; the initial DNP concentration was 78 mg/L(0.42 mM). Each data point in a profile represents the average of samples from duplicate flasks. Fig. 4 presents profiles for the same range of sodium ion concentration values, with a higher initial DNP concentration of 99 mg/L (0.53mM). The flask activated sludge concen-

trations, measured as MLSS, varied from 1,309 to 1,346 mg/L(dry weight). The DNP profiles in Fig. 5 and 6 are from the activated sludge cultures that were washed six times to achieve a lower sodium ion concentration of $9.3x10^{-5}$ mM, along with the higher concentrations of 9.33 and 93.3 mM. In Fig. 5 and 6, the initial DNP concentrations were 80mg/L and 100 mg/L, respectively. The MLSS concentrations for the six times-washed activated sludge cultures were 1,386 and 1,630 mg/L, respectively. In all the experiments, no significant difference in DNP degradation in different sodium ion concentrations was observed for the activated sludge cultures when either the single or the six-times washing method was used. Furthermore, the reduced sodium ion concentration of $9.3x10^{-5}$ mM had no significant effect when the initial DNP concentration was increased 20 % from 80 to 100 mg/L. In Fig. 4 and 6, the results from the experiments with the higher initial DNP concentration, there seems to be a slightly slower DNP degradation with the higher sodium ion concentration of 93 mM. It is possible that this effect was not a result of DNP toxicity, but rather the stress of the high salinity on the DNP-degrading bacteria.

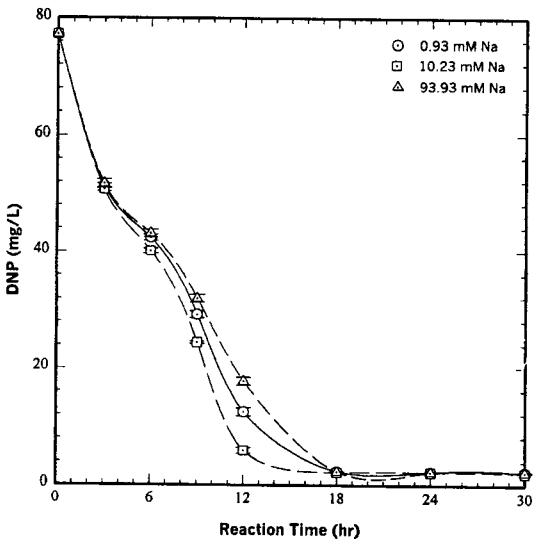

Fig. 3. DNP degradation profiles caused by single-washed activated sludge. Initial DNP concentration was 80 mg/L and MLSS was 1,309 mg/L.

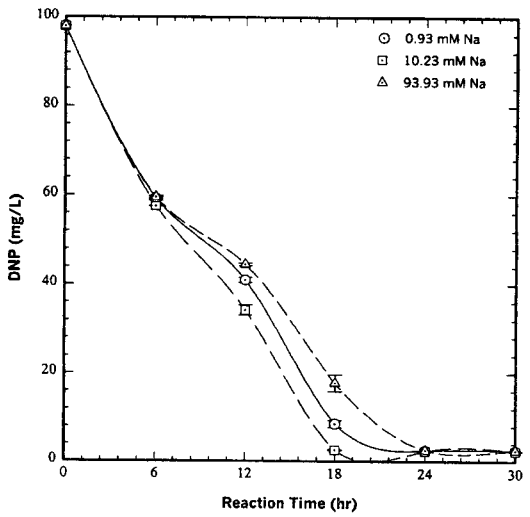

Fig. 4. DNP degradation profiles caused by single-washed activated sludge. Initial DNP concentration was 100 mg/L and MLSS was 1,346 mg/L.

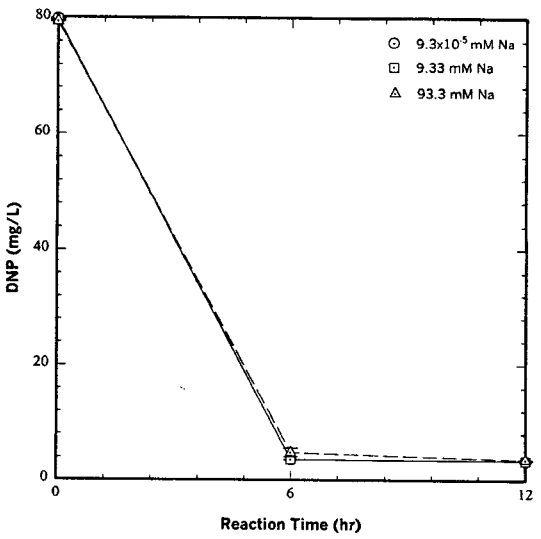

Fig. 5. DNP degradation profiles caused by six times-washed activated sludge. Initial DNP concentration was 80 mg/L and MLSS was 1,386 mg/L.

The DNP profiles for the degradation reactions with the pure culture isolates identified as *Nocardia asteroides* including sodium ion concentrations at 0 and 93.3 mM, are shown in Fig. 7. The initial

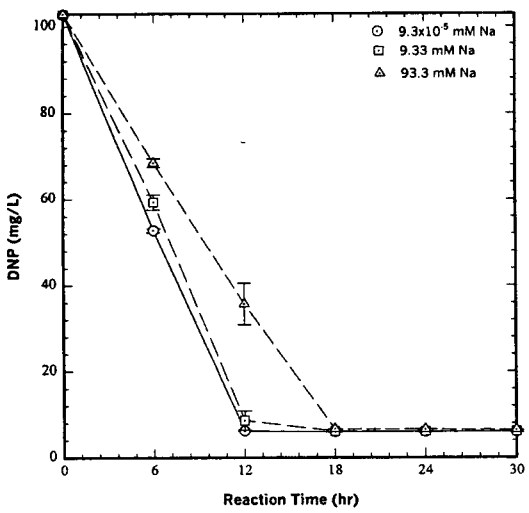

Fig. 6. DNP degradation profiles caused by six times-washed activated sludge. Initial DNP concentration was 100 mg/L and MLSS was 1,630 mg/L.

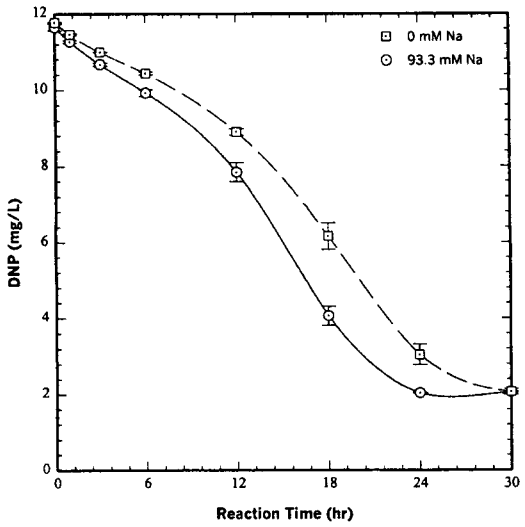

Fig. 7. DNP concentration profiles for flask degradation caused by *Nocardia asteroides* with either 0 or 93.3 mM sodium ions in the medium. Initial DNP concentration was 12 mg/L. Cell concentrations for all flasks were 3.75 mg/L.

DNP concentration for the pure culture experiments was 12 mg/L(0.064 mM). The cell concentration in each flask, for both the 0 and 93.3 mM sodium ion conditions, was 3.75 mg/L(dry weight). When the sodium ion concentration in the growth media for the DNP-degrading pure culture was 9.33 mM and the cell concentration was 12mg/L(dry weight), the time required to reach a DNP plateau value was significantly shorter for both the 0 and 93.3 mM sodium ion conditions(data not shown). As with the activated sludge experiments, each profile data point represents the average of the samples from duplicate flasks. According to these results, the isolate growing in the sodium-free medium appeared to degrade the DNP more slowly than the identical culture growing in the presence of 93.3 mM sodium ions. This difference may actually be underestimated because a relatively high sodium ion concentration of 93.3 mM was used - one seems to be associated with the reduced DNP degradation in the activated sludge flask experiments.

From an overall perspective, whereas the reduced medium sodium ion concentration of $9.3x10^{-5}$ mM seemed to have no significant effect on DNP degradation with an activated sludge mixed culture, the complete removal of sodium ions from the pure culture growth medium appeared to result in a slowing down of DNP degradation compared with the rate observed in the presence of 93.3 mM sodium ions. The results with the *Nocardia asteroides* isolate are consistent with the findings of other researchers in that a sodium gradient across the cytoplasmic membrane can be used to drive the oxidative phosphorylation of ATP in the presence of a protonophore such as DNP[4,6,7]. Accordingly, the uncoupling phenomenon caused by DNP may be overcome by establishing a sodium ion gradient across the membrane to drive the oxidation of DNP, especially in a pure culture. The oxidation of a carbon source is an exergonic process that provides the energy to create a gradient of protons(or maybe sodium ions) that is then used to drive the endergonic synthesis of ATP. Therefore, it is feasible that DNP can be degraded in high concentrations if the sodium ion concentration is high enough to facilitate a bacteria resistant to the toxic effects of DNP. The finding that there was only a slight difference in the effect of sodium ions on DNP degradation by an activated sludge mixed culture and by pure culture isolates suggests that one or more additional strains of microorganisms in the activated sludge mixed culture were mitigating the effect of the sodium ions.

4. Conclusions

The specific conclusions derived from this study are as follows :

1) A slower DNP degradation with a high sodium ion concentration was observed with the activated sludge cultures when both the single and six-times washing methods were used. It is possible that this effect was not the result of DNP toxicity, but rather the stress of the high salinity on the DNP-degrading bacteria.

2) A reduction in the growth medium sodium ion concentration to as low as 9.3×10^{-5} mM did not inhibit the degradation of either 0.43 mM(80 mg/L) or 0.54 mM(100 mg/L) DNP in acclimated activated sludge cultures.

3) The complete absence of sodium ions from the growth medium of a bacterial isolate from the activated sludge, identified as *Nocardia asteroides*, did retard the rate of degradation of 12 mg/L DNP, when compared with DNP degradation in a medium containing 93.3 mM sodium ions.

4) The effect of sodium ions on mitigating the uncoupling effect of DNP is expected to be more visible in pure culture DNP degradation than in an activated sludge mixed culture.

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