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Biodegradation of Dispersed Endicott Oil in Controlled Experiments

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ABSTRACT 299957:

We investigate aerobic biodegradation of dispersed Endicott oil in seawater at 15±0.5 °C in laboratory flasks. The objectives of the experiments were to (1) compare the biodegradability of chemically dispersed oil by Corexit 9500 with physically dispersed oil, and (2) determine whether the addition of nutrient affects the biodegradation rates of dispersed oil. The seawater samples (~ 6.5 g/L i.e. brackish water) were collected from Prince William Sound, Alaska. The biodegradation of Endicott oil was investigated for a period of 42 days under high nutrient (HN) (addition of 100 mg NO_3-N/L and 10 mg PO_4-P/L to background brackish water) and low nutrient (LN) (background brackish water) treatments. In the physically dispersed microcosms, oil biodegradation remained negligible for both HN and LN treatments. However, in the chemically dispersed oil microcosms, 24% and 14% of the total oil biodegraded in the HN (initial concentration= 0.304±0.095 g/L) and LN (initial concentration= 0.298±0.041 g/L) treatments within two weeks, respectively. These results demonstrated that the use of chemical dispersants coupled with nutrient addition can accelerate oil biodegradation. These findings can help develop better bioremediation strategies for addressing oil spills in the sea by focusing on simultaneous operations for rapid oil dispersion and stimulation of microbial growth through the availability of nutrients.

INTRODUCTION:

Chemical dispersants are commonly applied on oil slicks with the aim of dispersing oil into the water column to prevent the slick from reaching shorelines or harming organisms that inhabit surface waters (e.g., burds, mammals). A potential advantage for dispersion is the enhanced microbial degradation of the oil provided its concentration is not too high to engender toxicity. The removal of dispersed oil in seawater occurs primarily by biodegradation (Head et al., 2006; Yakimov et al., 2007; Prince et al., 2013; Venosa and Holder, 2013; McGenity, 2014). The effectiveness of chemical dispersants in mitigating marine spills depends on the extent to which the oil's bioavailability and biodegradation are enhanced relative to that of naturally occurring physically dispersed oil. The dispersion of oil in seawater largely depends on: the oil's physical properties (e.g. interfacial tension with water and oil viscosity), specific oil-dispersant combinations, oil dispersion effectiveness (i.e., the mass fraction of oil that becomes entrained in the water column as discrete small droplets), and the size of the oil droplets and their stability in the water column (i.e., ability of small droplets to resist coalescence into larger droplets that would tend to resurface).

The solubility of many oil components in water is very low, and oil biodegradation occurs mostly at the oil-water interface (Prince et al., 2013). Thus, the available surface area per unit volume of oil, the biodegradability of hydrocarbons in the dispersed droplets, and the abundance of microorganisms at the interface oil-water are important factors in determining the effects of chemical dispersants on oil biodegradation. Predicting the dispersion effectiveness for a range of crude oils and refined petroleum products with specific dispersants is rather challenging (Fiocco et al., 1999; Canevari et al., 2001; Mukherjee and Wrenn, 2011; Mukherjee et al., 2011). Nonetheless, it has been suggested that the attachment of bacteria to oil droplets could enhance alkane degradation (Zhang and Miller, 1992, 1993; Bruheim et al., 1997; Van Hamme and Ward, 2001), and the droplet-size distribution is expected to correlate with the oil biodegradation rate (Venosa and Holder, 2007).

The availability of oxygen and/or nutrients (nitrogen and phosphorus) can also accelerate oil biodegradation as reported by previous studies conducted in Prince William Sound (PWS) after the Exxon Valdez Oil Spill (EVOS) (Bragg et al., 1994; Boufadel et al., 2010; Sharifi et al., 2011). The literature contains a large range of NO_3-N (~ 2-10mg/L) concentrations that can support maximum oil biodegradation. For instance, Venosa et al. (1996) found that a concentration of about 1-2 mg N/L could support near optimum biodegradation activity. Boufadel et al. (1999) demonstrated that a concentration of 2.5 mg NO_3-N/L was sufficient to engender the maximum rate of heptadecane biodegradation. Phosphorus is also required to support microbial activity and oil biodegradation. Generally, a ratio of N:P of about 10:1 on a mass basis is typically recommended (Atlas and Bartha, 1973; Smith et al., 1998; Oh et al., 2003; Garcia-Blanco, 2004; Zahed et al., 2010).

In this study, we investigated aerobic degradation of Endicott oil, AK, in controlled laboratory conditions. The objectives of the experiments were to: (1) compare the biodegradability of oil chemically dispersed by Corexit 9500 to that of physically dispersed oil, and (2) determine whether the addition of nutrient affects the biodegradation rates of dispersed oil. The results showed that both chemical dispersion and nutrient availability enhanced oil biodegradation.

MATERIALS AND METHODS:

Brackish water sample collection and filtration

Brackish water samples were collected from Prince William Sound (PWS) south of the Valdez Narrows (61.04533N, 146.70190W), AK. Certified-clean amber-glass jugs were individually held under the water surface (10-15 cm below), filled by simply removing the cap, and closed before returning to the surface. The salinity was relatively low (~ 6.5 g/L). The water samples were filtered through a 10µm whatman paper filter using a filtering flask and a Buchner funnel (Figure 1A) in order to remove flagellates, ciliates, and other bacterial grazers that could increase the variability among the triplicate microcosms.

Preparation of chemically and physically dispersed oil in brackish water

Weathered Endicott oil, Ak, was physically or chemically dispersed in the filtered brackish water. A volume of 4.0 L of water was added to an aspirator bottle placed on a magnetic

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stirrer (Figure 1B). The stirring speed was adjusted to create a small vortex (~1/4 of water depth), and 20 mL of Endicott oil were added (i.e., oil-to-water ratio (OWR) = 1:200 (v/v)) into the vortex. Then, for the chemically dispersed oil bottle, 2 mL of dispersant (Corexit 9500) were added to the oil in the vortex (dispersant-to-oil ratio (DOR) = 1:10 (v/v)). The mixing speed was increased to draw the vortex all the way to the bottom of the bottle, such that air bubbles became entrained in the water column. The mixtures were stirred for 16 hours, and the suspension was allowed to settle for 30 minutes. Packs of ice were placed around the bottle to keep the mixtures at low temperature during the 16 h mixing time, thus limiting potential oil biodegradation. Two types of mixtures were generated : (1) brackish water with oil (no dispersant) referred to herein as water accommodated fraction (WAF), and (2) brackish water with oil and Corexit 9500 referred to herein as chemically enhanced water accommodated fraction (CEWAF).

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Oil biodegradation in WAF and CEWAF

Sealed microcosms were used (Fisher, 2005) to assess aerobic Endicott oil biodegradation in WAF and CEWAF. These microcosms were constructed using modified 250-mL wide-mouth, screw-cap Erlenmeyer flasks (Figure 2A). They included a CO_2 trap filled with a trapping solution (sodium hydroxide: NaOH), a sample port in the cap of the trap tubes for periodically removing or replacing the trapping solution, and a sidearm for controlled introduction of measured amounts of oxygen by attaching an oxygen-filled ground-glass syringe and allowing the pressure on the syringe barrel to equalize with the headspace gas pressure inside the microcosm. Carbon dioxide production was periodically measured by titration of the CO_2-trapping solution to the phenolphthalein endpoint (pH = 8.3) using sulfuric acid.

The oil biodegradation experiments were conducted in triplicate microcosms (a total of 54 including 6 as controls) for 42 days. 100 mL of WAF (0.0019 ± 0.0002 g of dispersed oil) or CEWAF (0.0301 ± 0.0066 g of dispersed oil) were added in each microcosm flask. The oil biodegradation was evaluated under high nutrient (HN) (100 mg NO_3-N/L and 10 mg PO_4-P/L + WAF or CEWAF) and low nutrient (LN) (WAF or CEWAF only, considering background nutrient in brackish water) treatments. In addition to the active columns, the experiments included 6 microcosms used as controls (3 WAF and 3 CEWAF + 0.5% w/v sodium azide, a bactericide). The microcosms were continuously agitated at 140 rpm by a shaker system and kept in the incubators at constant temperature (15±0.5 °C) (Figures 2B and C). Three independent replicate microcosms were sacrificed for every treatment at day 0 and every 2 consecutive weeks for monitoring total residual oil concentration (gravimetric method).

Nutrient concentrations in filtered brackish water, analyzed using a spectrophotometer (Thermo Scientific Evolution 201 UV-Visible) and test kits (HACH 2668000, 2429800, 2672245, 2106069, and 2742645), were considered as background nutrient concentrations of the WAF and CEWAF. Concentrations of nitrate (0.01-0.5 mg NO_3^-N/L), nitrite (0.002-0.3 mg NO_2^-N/L), ammonia (0.01-0.5 NH_3-N), total nitrogen (0.5-25 mgN/L), phosphorus reactive (orthophosphate) (0.02 to 2.50 mg PO_4^{3-}/L) and total phosphorus (0.06-3.50 mg PO_4^{3-}/L) were measured using the cadmium reduction, USEPA diazotization, salicylate, persulfate digestion, phosver 3 ascorbic acid and phosver 3 with acid persulfate digestion methods, respectively. Nutrient concentrations at time zero are reported in Table 1.

Determination of oil concentration

The total petroleum hydrocarbon (TPH) concentration was measured at time zero (i.e., after the mixing of oil with water) and at intervals of 2 weeks until the end of the experiments (6 weeks). It was obtained by gravimetric method after liquid-liquid extraction with dichloromethane (DCM). The extraction procedure included: (1) pouring the sample from the flask into a separatory funnel, (2) adding DCM and shaking vigorously for about 2 minutes, (3) allowing the DCM-oil phase and water phase to separate for about 5 minutes, and (4) collecting the DCM-oil phase in a clean glass beaker. These steps were repeated until the DCM phase became colorless i.e. no more oil remained in the sample. The extracted oil was transferred to a pre-weighed 24 mL glass vial and kept in the laboratory hood to allow evaporation of DCM. The extracted oil mass (M_{oil}) was derived from the weight difference between a vial with oil ($M_{vial.f}$) and an empty vial ($M_{vial.i}$). The significance of the results was statistically evaluated using analysis of variance (ANOVA) test at 95% confidence intervals (α=0.05).

Estimation of biomass growth

The biomass growth of alkane degraders, PAH degraders, and heterotrophic bacteria was estimated using the most probable number (MPN) method. The alkane degraders were incubated in Bushnell-Hass Broth (BHB) (Difco Laboratories, Sparks, MD) medium (0.2 g/L of $MgSO_4$; 0.02 g/L of $CaCl_2$; 1.0 g/L KH_2PO_4; 1.0 g/L of $(NH_4)_2HPO_4$; 1.0 g/L of KNO_3; 0.05 g/L of $FeCl_3$) and 5µL of hexadecane (as a carbon source). The PAH degraders were incubated in BHB and phenanthrene (10g/L), fluorene (1g/L), and dibenzothiophene (1g/L) in pentane as a carbon source. Yeast extract (0.2 g/L) was added to the PAH medium. The incubation medium for the heterotrophic bacteria was PTYG (0.25 g/L of peptone, 0.25 g/L of trypticase soy broth, 0.5 g/L of yeast extract, 0.5 g/L of glucose, 0.6 g/L of $MgSO_4 \cdot 7H_2O$, and 0.07 g/L of $CaCl_2 \cdot 2H_2O$).

RESULTS:

Concentrations of chemically dispersed and physically dispersed oil

The initial oil concentrations in the WAF microcosms were comparable at 0.020 g/L ± 0.003 for HN and 0.017 g/L ± 0.001 for LN (Table 2). They were also comparable in the CEWAF microcosms at an order of magnitude larger than in the WAFs : 0.304 g/L ± 0.095 for HN and 0.298 g/L ±0.042 for LN.

Temporal changes in oil concentrations

Temporal changes in total petroleum hydrocarbons (TPH) for HN and LN treatments are shown in Figure 3 (A, B, respectively). In the WAF microcosms, the TPH removal remained negligible for both HN and LN treatments during the 42 days of the experiment, indicating that oil biodegradation was limited by its bioavailability (initial total oil concentration was too low 0.020 g/L). Measured TPH in the control WAF microcosms at day 42 showed no evidence of oil biodegradation, and remained comparable with the TPH in the active microcosms (Figure 3 A and B). Within 14 days, the TPH removal in the CEWAF microcosms was higher in the HN (24%) (Figure 3A) than in the LN (14%) (Figure 3B). From day 14 to day 42, the TPH concentrations remained relatively unchanged in both HN and LN treatments, indicating that oil biodegradation was negligible. Measured TPH in the control CEWAF microcosms at day 42 showed, as expected, no evidence of oil biodegradation (Figure 3). These findings on oil removal suggest that :(1) chemical dispersion had substantially increased dispersed Endicott oil in

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brackish water and its subsequent aerobic biodegradation, and (2) the addition of nutrients to CEWAF Endicott oil in brackish water had further accelerated (a factor of ~ 2 in comparison with LN treatment) the rate of TPH removal within the first 14 days of the experiment. The ANOVA showed the observed difference in oil concentration between WAF and CEWAF at day 14 was statistically significant ($P < 0.001$), while it was not statistically significant ($P = 0.195$) for LN and HN.

Temporal changes in biomass

The temporal changes in the microbial population within the first 14 days of the experiments are reported in Table 3. The physically dispersed oil microcosms (WAF) revealed an increase in alkane degraders (AD) (3.28×10^2 count/mL to 5.52×10^3 count/mL) and heterotrophic bacteria (HT) (1.38×10^4 count/mL to 3.17×10^4 count/mL) in the HN and a rapid decrease in AD (2.93×10^2 to 0 count/mL) and HT (1.93×10^2 count/mL to ~ 2 count/mL) in the LN. The PAH degraders (PAHD) remained negligible for both HN and LN treatments. The chemically dispersed oil microcosms showed an increase in AD for both HN (2.23×10^4 to 2.46×10^6 count/mL) and LN (2.46×10^4 to 2.64×10^5 count/m). The population of HT also increased in both HN (4.39×10^5 to 6.14×10^7 count/mL) and LN (3.42×10^5 to 6.64×10^5 count/mL). An increase in PAHD was observed only in the HN although this population remained relatively low (1.00×10^0 to 1.80×10^1 count/mL).

DISCUSSION:

The experiment was conducted to evaluate the effects of physical and chemical dispersion (using Corexit 9500) along with nutrient availability (nitrogen and phosphorus) on aerobic biodegradation of oil. The physical dispersion resulted in an initial oil concentration that was one order of magnitude lower than that of the chemical dispersion. These results indicate that the applied dispersant and mixing conditions significantly enhanced the oil dispersion into brackish water. The yield of CEWAF increased by a factor of 2 in comparison with a yield of 0.151 ± 0.004 g/L obtained previously by applying a vortex depth to the water-oil mixture column of ~ 20-25%. The current experiment also significantly reduced the settling time from 6 to 0.5h. Biodegradation in the CEWAF led to a removal of up to 24% of total petroleum hydrocarbon-TPH. The removal of TPH was negligible in the WAF. The concentration of oil needed to support effective biodegradation was apparently sufficient in the CEWAF and too low in the WAF. Our results are supported by previous studies demonstrating an increase in dispersed oil and its biodegradation when applying Corexit 9500 (e.g., Venosa and Holder, 2007).

Assessing the effectiveness of bioremediation for the Exxon Valdez oil spill, Bragg et al. (1994) argued that the amount of oil and nitrogen availability were among the most significant factors determining the rate of biodegradation. These two factors have apparently played a major role in the oil biodegradation observed in our study. In addition to the effects of chemical dispersion on oil bioavailability and biodegradation, the availability of nutrients substantially enhanced oil biodegradation (Cf. Figure 3). The TPH removal in the nutrient-added (HN) treatment (24%) was almost twice of that of the low nutrients (LN) treatment (14%) during the first 14 days of the experiment. The greater oil removal in the HN CEWAF treatment provided evidence on the influence of nutrients on the rate of oil biodegradation. The availability of a sufficient amount of nutrients appeared to be the primary driver for microbial growth and oil

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biodegradation. The microbial population growth was substantial higher in the HN than the LN treatments (Cf. Table 3). Our findings are consistent with the observations of Venosa et al. (1996) who demonstrated that the biodegradation rate of total alkanes and total aromatic was significantly higher in the nutrient-added treatment in comparison with the non-nutrient-added treatment.

The large variability observed in the oil concentration as depicted by the standard deviation could explain why the difference between the LN and HN treatments of the CEWAF was not statistically significant. ANOVA has been commonly used to determine the statistical significance of results of different treatments from oil biodegradation studies (e.g., Venosa and Holder, 2007). Applying ANOVA, a difference is “statistically significant” when it is large enough compared with its standard deviation. Thus, when a standard deviation is very small**,** a small difference can be “statistically significant”. Similarly if the standard deviation is large, only a huge difference will be “statistically significant”. This suggests that “statistically significant” or “not statistically significant” results may not have any practical importance (Navidi, 2011). In our experiments, the overall percent of oil removal appeared to be a more practical way to look at the difference between the HN and LN treatments. For instance, nutrient supplements increased by a factor of ~ 2 the amount oil removal, which should make a huge difference in a case of oil spill responses. Note that extensive mechanical operations after the Deepwater Horizon spill in the Gulf of Mexico have led to only 3% of oil removal by skimming (NOAA, 2010). In our experiment, the observed difference between nutrient and non-nutrient supplementation was 10%.

The brackish water samples were filtered to remove flagellates (size 14.3-20 µm in length and 1.5-1.8 µm in thickness), ciliates (size 20-400 µm), and other bacterial grazers that may increase the variability among the triplicate flasks, while ensuring a sufficient starting population of bacteria. The water filtration could have changed the structure of the microbial communities, reduced predation and inflated the biodegradation rates. However, as reported by Gertler et al. (2010), the presence of predators could also increase oil emulsification, prevent the microbes from entering a stationary phase, and stimulate microbial growth through the excretion of beneficial microbial growth factors (e.g., vitamins, amino acids). This suggests that the presence of predators and predation processes could also increase the biodegradation rates. Therefore, the biodegradation rate that we observed in our experiments may not have been overestimated.

Some limitations of our findings result from the fact that the controlled experimental conditions are clearly different from in situ conditions. If spill occurs in coastal brackish waters, a range of physical, chemical and biological factors are subject to dynamic changes and interactions that could influence oil biodegradation processes. For instance, the action of waves could lead to a rapid dilution of oil, applied dispersants and nutrients in huge volume of brackish water within hours or days. Another limitation is the observed large variability in the results. This variability could be due to the initial differences in each flask although similar procedures were applied. Uncertainties in oil extraction with DCM could also contribute in the observed variability. Nonetheless, our findings show the potential for accelerating oil biodegradation using together a chemical dispersant and nutrient supplementation.

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CONCLUSION:

Under similar mixing conditions, the application of Corexit 9500 at a dispersant-to-oil ratio of 1/10 v/v and oil-to-water ratio of 1:200 v/v has resulted in an order of magnitude increase in the initial Endicott oil concentration in comparison to its physical dispersion. Our results indicate that the dispersant was effective in increasing oil biodegradation. While no TPH removal was observed in the physically dispersed oil microcosms, significant TPH removal (up to 24%) occurred in the chemically dispersed oil microcosms. We have also shown that the addition of nutrients (N and P) increases oil biodegradation by a factor of ~ 2 in comparison with its biodegradation in background brackish water nutrient conditions. We conclude that the application of dispersants combined with nutrients can substantially enhance oil bioremediation in brackish water.

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Table Captions

Table 1. Nutrient concentration in high-nutrient and low-nutrient flasks at time zero

Table 2. Dispersed oil concentration in high-nutrient (HN) and low-nutrient (LN) treatments after 16 hours of mixing and 30 minutes of settling.

Table 3. Temporal changes in biomass

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Table 1. Nutrient concentration in high-nutrient and low-nutrient flasks at time zero

Nutrients	High-Nutrient Flasks	Low-Nutrient Flasks
NH_4-N (mgN/L)	0.052±0.005	0.052±0.005
NO_3-N (mgN/L)	100.012±0.001	0.012±0.001
NO_2-N (mgN/L)	0.010±0.0051	0.010±0.001
Total N (mgN/L)	~100.5	< 0.5 (BDL)
Orthophosphate (mgPO_4-P/L)	10.106±0.009	0.106±0.009
Total P (mgPO_4-P/L)	10.235±0.017	0.235±0.017

BDL – below detection limit

Table 2. Dispersed oil concentration in high-nutrient (HN) and low-nutrient (LN) treatments after 16 hours of mixing and 30 minutes of settling.

Microcosms	High-Nutrient	Low-Nutrient Flasks
Chemically dispersed (CEWAF)	0.304 g/L ± 0.095	0.298 g/L ±0.042
Physically dispersed (WAF)	0.020 g/L ± 0.003	0.017 g/L ± 0.001

WAF : Physically dispersed oil microcosms
CEWAF: Chemically dispersed oil microcosms

Table 3. Temporal changes in biomass

	Day 0 (count/mL)	Day 14 (count/mL)
Alkane Degraders		
WAF-LN	2.93×10^2	0
WAF-HN	3.28×10^2	5.52×10^3
CEWAF-LN	2.46×10^4	2.64×10^5
CEWAF-HN	2.23×10^4	2.46×10^6
PAH degraders		
WAF-LN	0	0
WAF-HN	0	0
CEWAF-LN	0	0
CEWAF-HN	1.00×10^0	1.80×10^1
Heterotrophic bacteria		
WAF-LN	1.93×10^2	2×10^0
WAF-HN	1.38×10^4	3.17×10^4
CEWAF-LN	3.42×10^5	6.64×10^5
CEWAF-HN	4.39×10^5	6.14×10^7

WAF : Physically dispersed oil microcosms HN: High nutrient
CEWAF: Chemically dispersed oil microcosms LN: Low-nutrient

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Figure Captions

Figure 1. A) Vacuum filtration of seawater samples through a 10 µm paper filter before oil dispersion; and B) Mixture of seawater (4L) with oil and dispersant in an aspirator bottle (mixing time = 16 h.; settling time = 30 min.; Oil-to-Water Ratio = 5:1000 (v/v); Dispersant-to-Oil Ratio = 1:10 (v/v)).

Figure 2. Experimental set-up: A) Schematic diagram of microcosms used to measure oil biodegradation; B) Incubators for controlled temperature; and C) Microcosm on shakers with a rotational speed of 140 rpm inside the incubators at the temperature of 15±0.5 °C.

Figure 3. Variation in total petroleum hydrocarbons (TPH) as a function of time for both high nutrients (HN, Panel A) (background seawater + 100 mg NO_3^-N/L and 10 mg PO_4^{-3}P/L), and low nutrients (LN, Panel B) (background seawater only). Hollow symbols represent TPH concentration in WAF microcosms (i.e. no addition of dispersant). Filled symbols are for CEWAF microcosms (i.e., addition of the dispersant Corexit 9500). Error bars represent one standard deviation based on triplicates. TPH concentrations at the end of the experiments in the control microcosms are shown for WAF (hollow square) and CEWAF (filled square).

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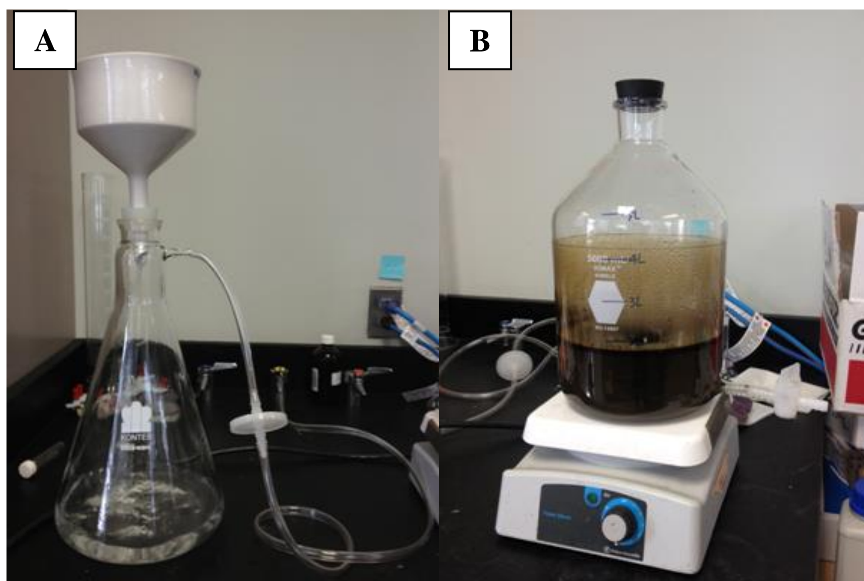

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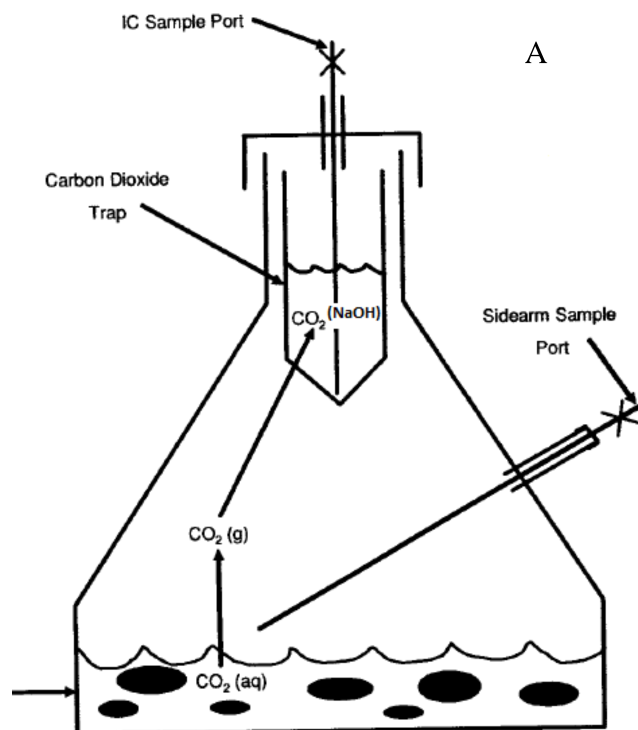

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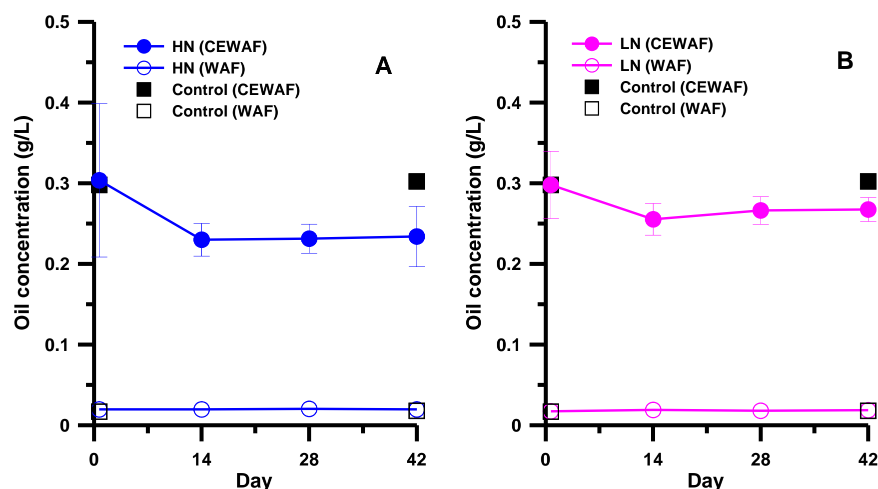

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