AN ASSESSMENT OF DNA AS A RESOURCE FOR AQUATIC BACTERIOPLANKTON

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ABSTRACT

Dissolved DNA (dDNA) is a potentially important source of energy and nutrients for bacteria in the natural environment. We isolated bacteria from Eel Pond, Woods Hole, MA where DNA was the sole source of carbon (C), nitrogen (N), and phosphorus (P). Colony forming units (CFU's) were significantly higher when DNA was supplied as degraded (mono- and oligonucleotides) versus intact (< 2000 base pairs, double stranded) DNA. 16S rDNA analysis revealed that there were differences in the structure of cultivable bacterial communities when grown on degraded and intact DNA, with higher diversity on degraded DNA. From our primary enrichments, we isolated bacteria from degraded DNA and then measured their growth rates in broth media using intact and degraded DNA sources. In general, we found that growth rates were higher on degraded DNA than intact DNA, which agreed with some direct measurements of DNA consumption for select isolates. We also developed an ethidium bromide staining assay to assess the degree of DNA depletion by different environmental isolates. We found that there was variation in, what may be, exonuclease activity that is used to breakdown large molecular weight DNA. Our results suggest that isolates originally from intact DNA depleted DNA from larger areas than isolates from degraded DNA. As far as we know, this is one of the first studies demonstrating that dDNA can be resource for certain phylotypes of marine bacterioplankton. Future studies should examine the relative importance of exoenzyme production and competency-mediated uptake of DNA by different species of bacteria.

INTRODUCTION

"Heterotrophic" bacteria play important roles in the functioning of aquatic ecosystems. The abundance and metabolic activities of aquatic bacteria are largely determined by the availability of dissolved organic resources. One potentially important, but overlooked resource is dissolved (i.e., free) DNA. Concentrations of dDNA range from 0.5 – 88 μg/L (Paul et al. 1987) and comprise a large fraction of the carbon (C), nitrogen (N), and phosphorus (P) pools in aquatic environments (Matsui et al. 2001). For example, dDNA can account for up to 28% of the total P in some freshwater ecosystems (Siuda and Chrost 2000). A number of studies have documented that some bacteria (e.g., *E. coli*) are capable of using dDNA as a resource (e.g., Finkel and Kolter 2001). However, to our knowledge, no one has investigated the phylogenetic identity and diversity of DNA-consuming bacteria in natural aquatic communities.

We hypothesized that marine heterotrophic bacterioplankton vary in their ability to utilize dDNA as a resource. We conducted enrichment experiments on bacterioplankton from Eel Pond, Woods Hole, MA to assess whether there are differences in the diversity of cultivable microorganisms to use dDNA as a sole C, N, and P resource. Additionally, we hypothesized that the structure of DNA would influence the ability of bacterioplankton to grow on dDNA as a resource. To test this hypothesis, we exposed marine isolates to small, degraded DNA and larger, intact DNA on both agar and broth media.

The ability for bacteria to use dDNA may be influenced by two mechanisms involved in DNA uptake. Numerous researchers interested in lateral gene transfer have focused on the uptake of intact DNA via competency genes (e.g., Finkel and Kolter 2001). However, DNA may also be

degraded with the aid of exonucleases prior to being taken up by bacteria. We hypothesized that bacterioplankton from Eel Pond vary in their exonuclease capacity. We tested this hypothesis by developing an enzyme assay that detects the depletion of intact DNA around bacterial colonies on agar plates.

MATERIALS AND METHODS

Field Sampling – We obtained water samples from Eel Pond in Woods Hole, MA on 12 July 2004. Eel Pond is a small impoundment open to the harbor. We collected water with a peristaltic pump at one location, from three depths (0.6, 2.4, 5.2 m); Eel Pond had a maximum depth of 5.5 m at our sampling location. In addition to collecting water, we measured salinity, dissolved oxygen, and conductivity using a YSI multiprobe water quality checker. Water samples from multiple depths were pooled and transported immediately back to the laboratory. We stained a water sample with DAPI and quantified bacterial abundance using epifluorescence microscopy.

dDNA media– We made DNA media with sodium salt, double-stranded DNA derived from salmon testes with a reported size of < 2000 base pairs (Sigma, D-1626). Using this DNA source, we created two types of dDNA: intact and dissolved. We created an intact DNA source by directly dissolving 1 g of DNA into 100 mL of distilled water. We created a degraded source of DNA by dissolving 1 g of DNA into 100 mL of distilled water with 7000 units of DNAse I (Roche 104 132) and DNAse buffer (200 μM Tris pH 8.3, 500 μM KCl, 10 μM $MnCl_2$) for 3 hours at 37°C. We stopped the DNAse reaction by heating at 70°C for 20 minutes. We also added heat-killed DNAse (100°C for 1 hour) and DNAse buffer to the intact DNA to control for the compounds that were used in making degraded DNA. We confirmed the effects of DNAse activity by evaluating DNA size by running an electrophoretic agarose gel (Fig. 1). We then added either intact or degraded DNA to autoclaved, artificial seawater media at a final concentration of 1 g DNA/L. The sea water media consisted of 30 g/L of sea salts (Sigma), 10 mM Hepes buffer, and trace metal solution (course handout). For the DNA plates, we added washed agar to the seawater prior to autoclaving. The washing process consisted of rinsing the agar seven times with distilled water, one time with 70% ethanol, and one time with acetone, followed by aeration at 40°C for three days. Control media consisted of all of the above ingredients with the exception of DNA.

Primary enrichments – We made a dilution series of Eel Pond water and plated 100 μL onto the different DNA and control treatments. We incubated the plates at 30°C and monitored colony formation daily. On day 6 we used a set of replicate plates for plate wash PCR (see below). On day 10 we calculated the number of colony forming units (CFU's) for replicate plates of each treatment. We used a one-way ANOVA and Tukey's HSD to test for differences in CFU's between DNA treatments.

Community structure of bacteria growing on DNA media – We extracted genomic DNA from intact and degraded DNA-containing agar plates using a plate wash technique to gather information on the diversity of bacteria that grew on DNA media. We extracted DNA using the Ultraclean Fecal DNA isolation kit for fecal samples (MoBio Laboratories). Briefly, 2 ml of

bead solution was pipetted onto the surface of each plate. We spread the bead solution over the cells, collected the liquid in a 2 ml Fecal Dry bead tube, and proceeded according to the manufacturer protocol. With the extracted DNA, we constructed two 16S rDNA clone libraries for each DNA source (degraded and intact). We amplified 16S rRNA genes using bacterial universal primers (8f and 1492r), screened by agarose gel electrophoresis. We then cloned the PCR products using the Invitrogen TOPO TA® cloning kit. A total of 96 clones from each library were sequenced, and subsequently analyzed using ARB phylogenetic software. Statistical differences among the two different libraries were determined using Libshuff software.

Identity of selected isolates – A total of 37 isolates (19 from the "degraded" and 18 from the "intact") were randomly obtained from the primary enrichments and subsequently isolated on solid agar media containing the same initial DNA source of enrichment (intact or degraded). After growth, each isolate was identified using 16S rDNA sequencing. To do this, individual colonies were picked and transferred into 20 µL of 0.05% Triton X-100 solution, boiled for five minutes, and then centrifuged to separate the cell material. 1 µl of the resulting supernatant was used as a template in a 25µl PCR reaction. PCR products were screened by agarose gel electrophoresis and sequenced, after cleaning with the ExoSAP-IT® kit (USB Corporation). Sequences were analyzed on-line (www.ncbi.nlm.nih.gov/BLAST) using BLAST.

Growth curve and dDNA experiments – Phylogenetically-identified isolates from the degraded DNA treatment were used in a set of growth curve experiments on liquid media. This allowed us to confirm that the isolates could exclusively grow on DNA, and not just agar + DNA. The composition of the liquid medium was the same as described above (see ***dDNA media***). For each isolate, we added intact or degraded DNA to autoclaved test tubes (15 mL) and then inoculated the tubes with a homogenous solution of cells (100 µl of a 0.7% NaCl suspension). After inoculation, we incubated the tubes in an upright shaker at 30°C. We monitored growth as absorbance at 600 nm (OD600) with a spectrophotometer, specifically adapted to hold test tubes. We grew each isolate on degraded and intact DNA in triplicate. We determined the effects of DNA type (intact vs. degraded) using repeated measures ANOVA (SAS PROC MIXED with an AR(1) covariance matrix).

dDNA consumption – We quantified DNA consumption within selected broth cultures spectrophotometrically by measuring absorbance at 260 nm. A 50 µL aliquot of the broth culture was collected at the beginning and end of the experiment. The aliquots were centrifuged before spectrophotometric reading to reduced interference by cellular material. The supernatant was analyzed under a SmartSpec Plus spectrophotometer (BIORAD). DNA concentrations were determined assuming a conversion factor of 50µg/mL for intact DNA and 20 µg/mL for degraded DNA. We used different conversion factors because the double stranded DNA absorbs light differently than the oligonucleotides in the intact DNA treatment.

Exonuclease activity – We evaluated DNA depletion, which was presumably due to exonuclease activity, by soaking agar plates in 1X TBE bath with ethidium bromide at a 100 µg/L final concentration for two minutes. After soaking, the plates were rinsed in a 1X TBE bath for one minute before taking pictures under fluorescent light with a transilluminator adapted with image analysis software. We qualitatively scored the degree of DNA depletion with the following scores: 1 = 0-25%, 2 = 26-50%, 3 = 51–75%, 4 = 76-100%. We then tested whether bacteria

originally isolated from intact DNA enrichments produced larger zones of clearing than bacterial originally isolated from degraded DNA. We tested this using a contingency analysis (JMP).

RESULTS AND DISCUSSION

Field Sampling – The physical and chemical characteristics of Eel Pond are described in Table 1. In general, salinity, temperature, and conductivity of this waterbody were uniformly distributed with depth. Dissolved oxygen concentration decreased with depth to a low of 3.6 mg/L. We determined that the total abundance of DAPI-stained bacteria was 1.3 (± 0.71 SE) x 10^7 cell/mL.

Primary enrichments – The number of CFUs was significantly higher in degraded than intact DNA plates (ANOVA, $p<0.0001$, Fig. 2). There were more colonies on the degraded and intact DNA plates than on the control plates (Fig. 2). We conclude that very few bacteria are capable of growing on agar alone in our experiments. Our results also suggest that there are differences in the quality of the two different DNA sources. In particular, more colonies formed on the degraded DNA, supporting our hypothesis that the structure of dDNA affects the capability of bacterioplankton to use DNA as resource. This result does not however indicate that bacteria were only using DNA as a resource on our plates. In fact, we observed that some isolates were agarolytic, making large depressions in the plate. Thus, it is possible that the bacteria obtained nitrogen and phosphorus from the DNA and some carbon from the agar.

Community structure of bacteria growing on DNA media – Results from the plate wash clone library on intact and degraded DNA are shown in Fig. 3. On degraded DNA, bacteria were related to the genera *Pseudoalteromonas*, *Alteromonas* and *Flexibacter*, whereas bacteria growing on intact DNA were limited to the genera *Pseudoalteromonas* and *Alteromonas*. Libshuff results suggest that the two libraries were significantly different from one another ($P < 0.029$) and that bacteria from the intact DNA treatment were a subset of those growing on degraded DNA. Our results suggest that there are some taxa that are capable of using both degraded and intact DNA, but there are some bacteria that were limited to growing on the oligonucleotide DNA.

Admittedly, there is cultivation bias in our study. As such, we may be grossly underestimating the number and diversity of bacterioplankton that are capable of using DNA as a resource. Nevertheless, this work is useful because there are very few (if any) studies that have attempted to characterize the identity of DNA-consuming bacterioplankton.

Identity of selected isolates – Sequenced isolates from the intact DNA plates belonged to the genera *Alteromonas* and *Pseudoalteromonas*, whereas isolates from the degraded DNA plates also contained bacteria from the genera *Microscilla* and *Vibrio*.

Growth curve and dDNA experiments – Isolates originally from the degraded DNA plates had positive growth when inoculated broth containing degraded DNA; only one isolate did not grow (*Pseudoalteromonas* sp. MD 213). In the absence of agar, our broth experiments confirm that bacterioplankton can grow well on degraded dDNA.

In contrast, 37% (7/19) of the isolates originally from the degraded DNA plates failed to grow on intact DNA in broth media over the duration of short-term experiment. This suggests that there may be constraints or costs associated with the consumption of large molecular weight DNA. Furthermore, 56% of the isolates originally from degraded DNA plates experienced lower growth intact versus degraded DNA in broth media (Figs. 3-6).

dDNA consumption – We measured consumption rates of intact and degraded DNA for three *Vibrio* strains. We chose these strains because they had higher growth rates and produced turbid cultures in the broth tubes. As such, we expected that these isolated would demonstrate some of the highest rates of DNA consumption. All three *Vibrio* strains consumed large quantities of DNA (Fig. 7). *Vibrio pelagius* consumed intact and degraded DNA at the same rate (Fig. 7a). In contrast, *Vibrio vulnificus* and *Vibrio* sp. MD208 consumed degraded DNA more rapidly than intact DNA (Fig. 7b and 7c). Together, these results confirm that bacterioplankton were capable of consuming DNA and that species vary in their ability to use different types of DNA (i.e., degraded vs. intact).

Exonuclease activity – Initial observations of our primary enrichments indicated that there were zones of DNA depletion around colonies. We stained one of these plates with ethidium bromide to make the zones of DNA depletion more apparent (Fig. 8). We further developed this technique (Fig. 9) and tested this on all of the isolates from our primary enrichments (intact and degraded treatments). Our results suggest that there is large variation in the ability of bacteria to deplete intact DNA, independent of colony size. Furthermore, our results suggest that isolates originally from intact and degraded DNA enrichments vary in the degree of DNA depletion (Fig. 10, contingency analysis, likelihood ratio, $P = 0.07$). Qualitatively, it appears that intact isolates had a tendency to have more class 4 clearing zones, while degraded isolates had a higher proportion of class 1 clearing zones.

It is important to note that our assay does not definitively measure enzyme activity. Its possible that zones of depletion are due to diffusion followed by bacterial uptake. In addition, we might expect similar results if bacteria were consuming DNA via direct uptake without extracellular degradation. One such mechanisms could be through natural competency of bacterioplankton. For example, Finkel and Kolter (2001) found that a number of genes involved in the direct uptake of DNA by *E. coli* and that knock outs of these genes resulted in lower bacterial growth when DNA was the only resource. Finkel and Kolter (2001) also found that homologous competency genes were found in other bacteria. Future studies should evaluate the relative importance of exoenzyme production and competency-mediated uptake of DNA by different species of bacteria.

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Table 1. Physical and chemical characteristics of Eel Pond in Woods Hole, MA on 12 July 2004.

Depth (m)	Salinity (ppt)	Dissolved Oxygen (mg/L)	Temperature (°C)	Conductivity (mS)
0.6	32.2	7.3	23.5	47.8
2.4	32.2	7.5	22.1	46.6
5.2	32.2	3.6	21.3	45.7

Figure 1. Gel electrophoresis demonstrating the relative differences of the two types of dDNA used in enrichment and growth curve experiments. L=ladder; I = intact dDNA; D = degraded DNA.

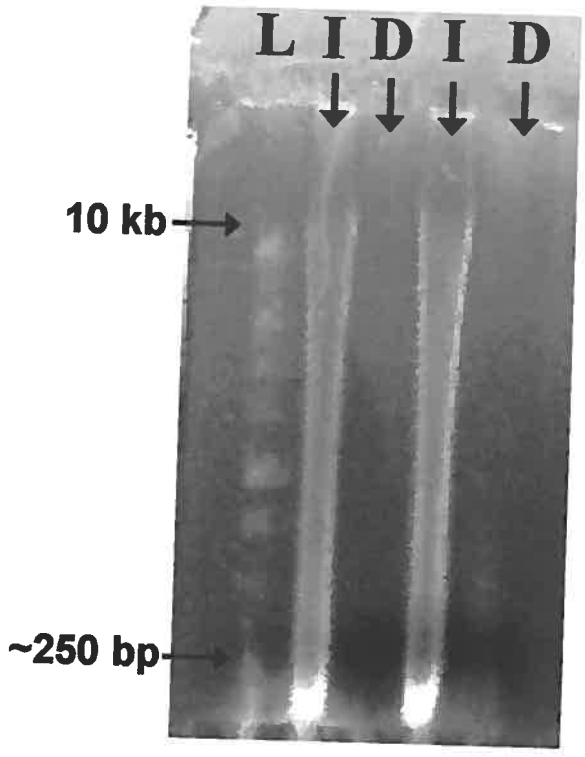

Figure 2. Colony forming units (CFU's) on primary enrichment plates containing intact and degraded dDNA. Data equal mean ± SEM.

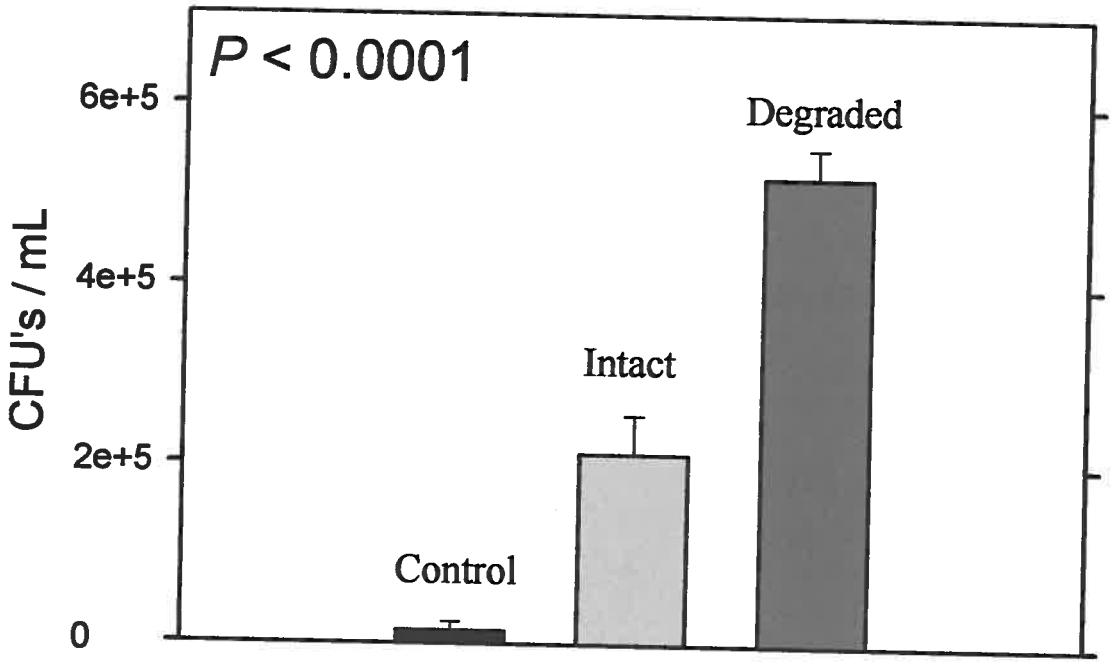

Figure 3. Phylogenetic tree showing the relationship of plate wash communities on degraded and intact dDNA.

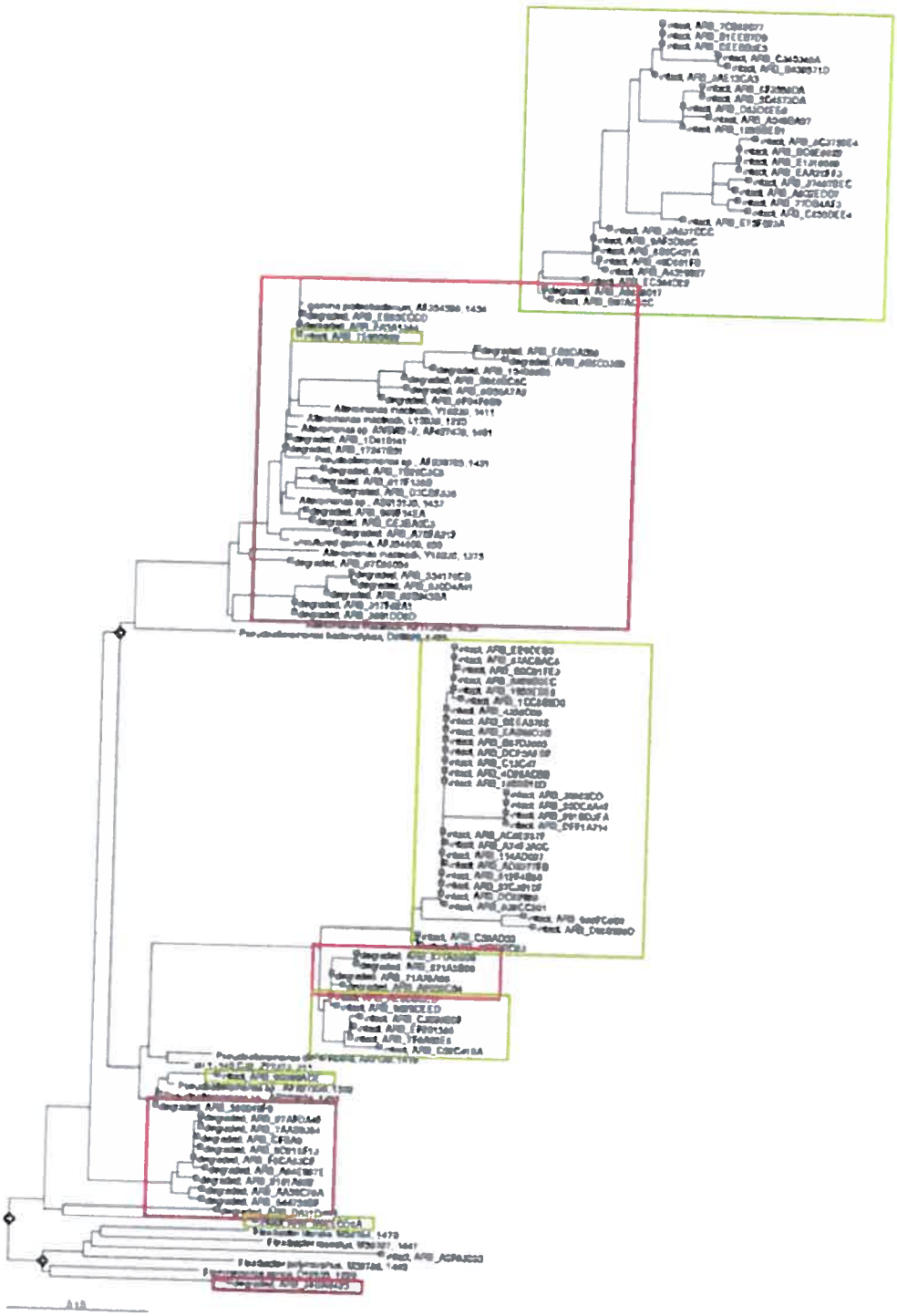

Figure 3. Growth curves experiments on *Alteromonas* isolates. We measured OD 660 over time on isolates originally from the degraded dDNA enrichment plates in broth media with intact (●) and degraded (○) dDNA. Data equal mean ± 1 SD. Probability (*P*) values refer to results from repeated measures ANOVA.

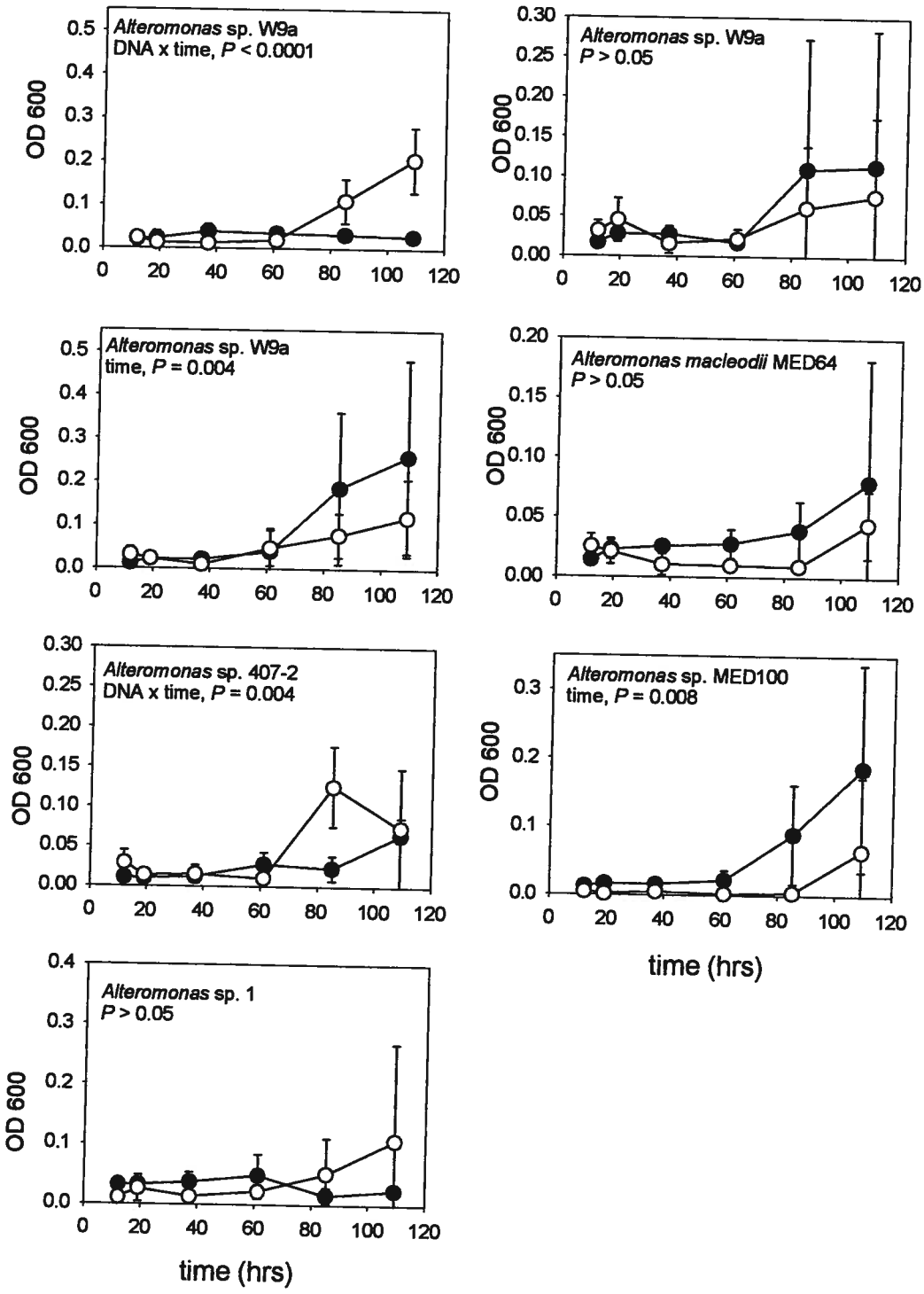

Figure 4. Growth curves experiments on *Pseudoalteromonas* isolates. We measured OD 660 over time on isolates originally from the degraded dDNA enrichment plates in broth media wi intact (●) and degraded (○) dDNA. Data equal mean ± 1 SD. Probability (*P*) values refer to results from repeated measures ANOVA.

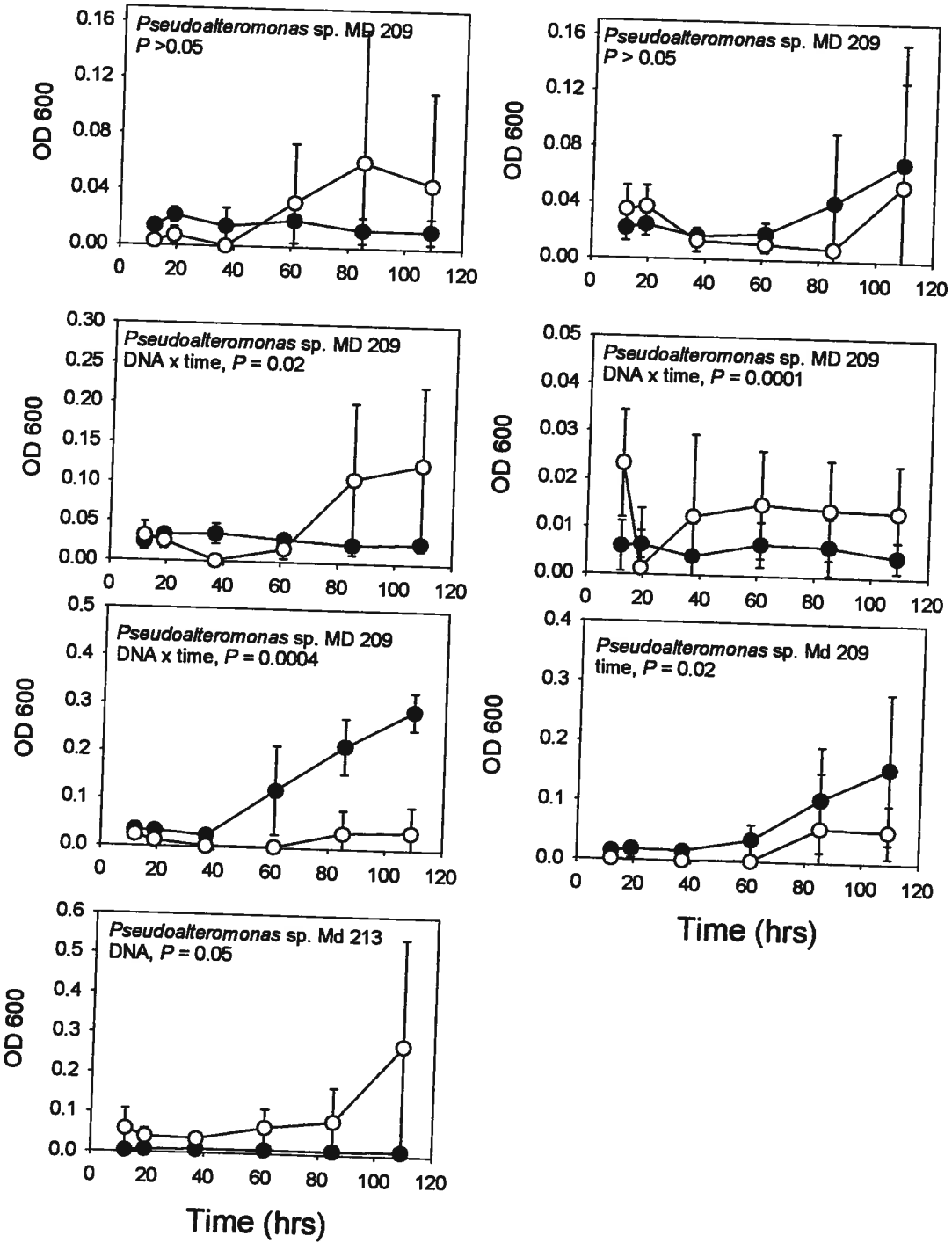

Figure 5. Growth curves experiments on *Vibrio* isolates. We measured OD 660 over time on isolates originally from the degraded dDNA enrichment plates in broth media with intact (●) and degraded (○) dDNA. Data equal mean ± 1 SD. Probability (*P*) values refer to results from repeated measures ANOVA.

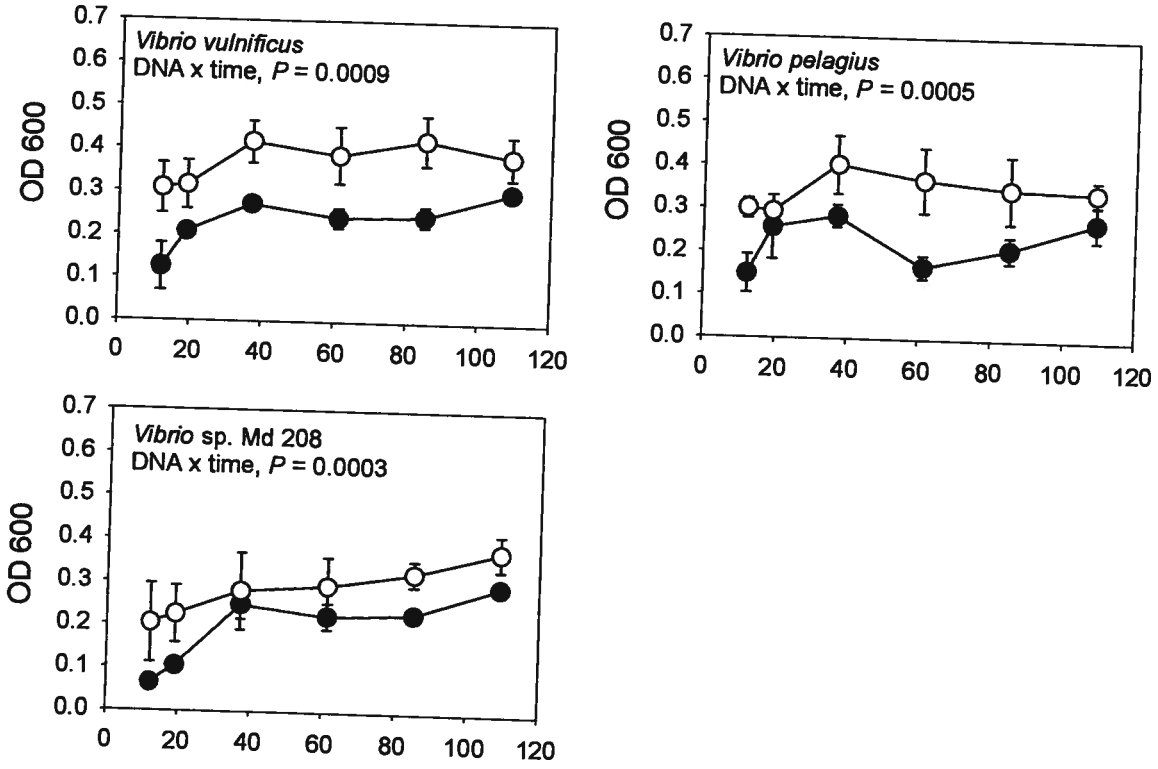

Figure 6. Growth curves experiments on *Microscilla* isolates. We measured OD 660 over time on isolates originally from the degraded dDNA enrichment plates in broth media with intact (•) and degraded (○) dDNA. Data equal mean ± 1 SD. Probability (*P*) values refer to results from repeated measures ANOVA.

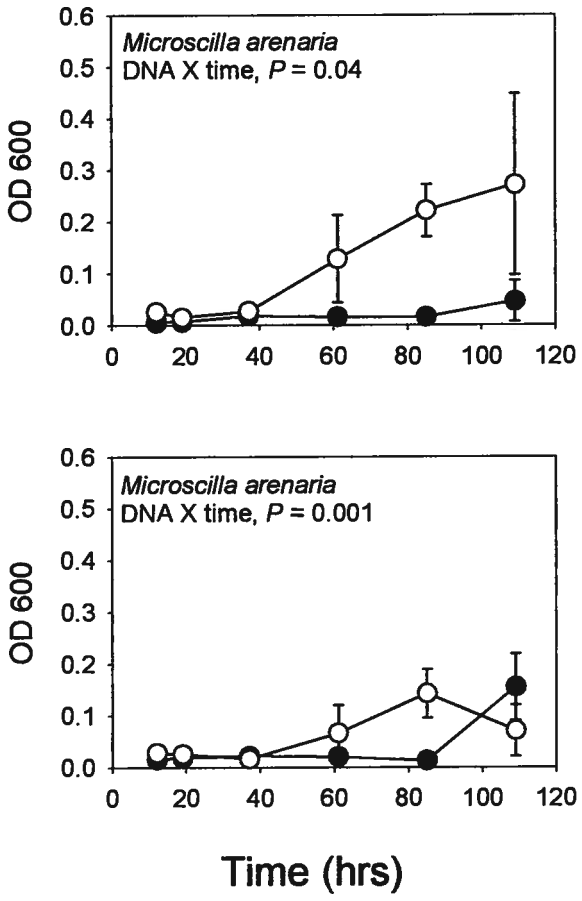

Figure 7. Rates of dDNA consumption for three *Vibrio* strains isolated from degraded DNA enrichments in broth containing degraded and intact dDNA. Data equal mean ± SEM. Probability (*P*) values are from t-tests.

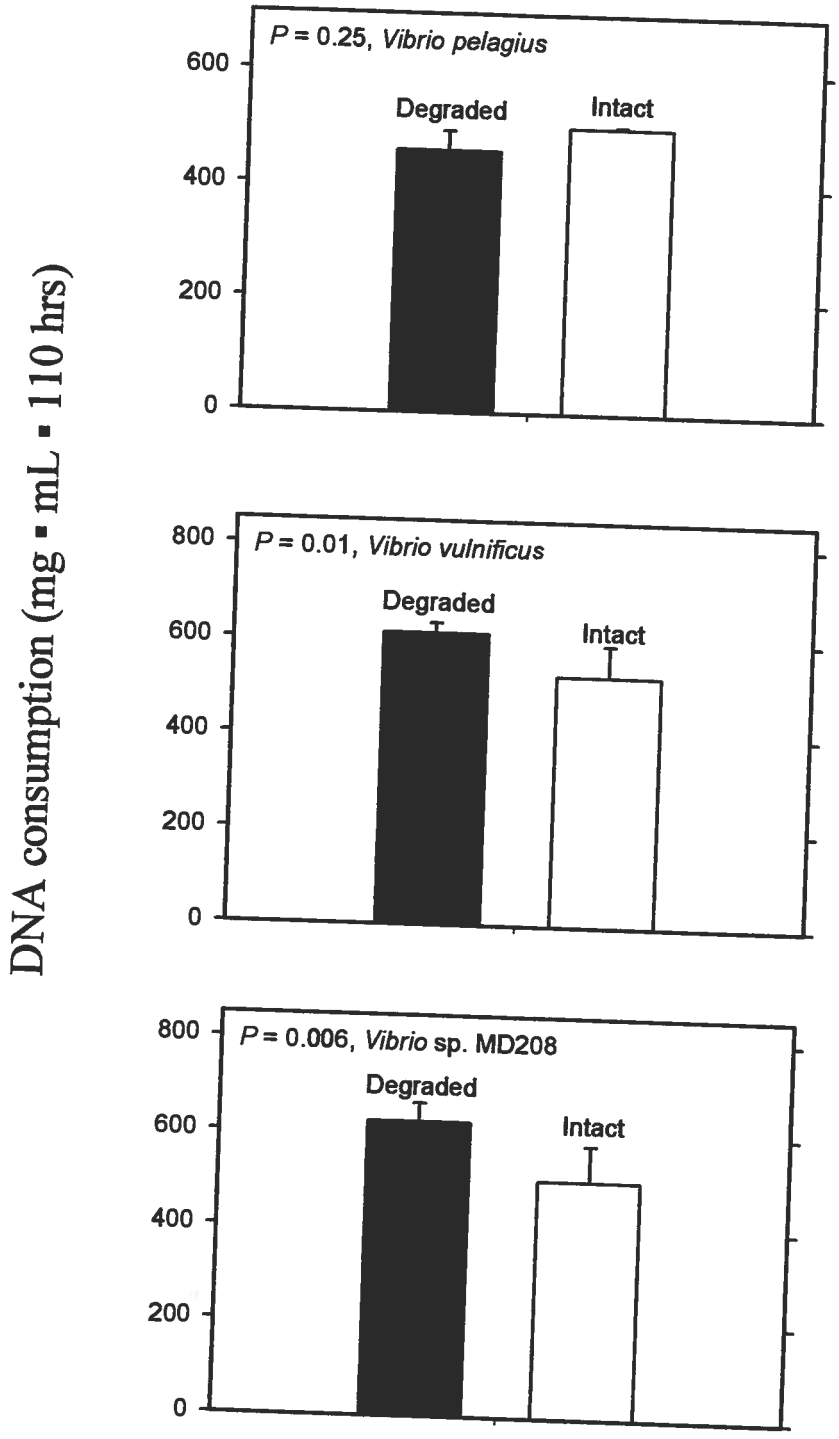

Figure 8. Bacterial colonies from primary enrichments on intact dDNA. We observed dark zones of dDNA depletion after staining with ethidium bromide.

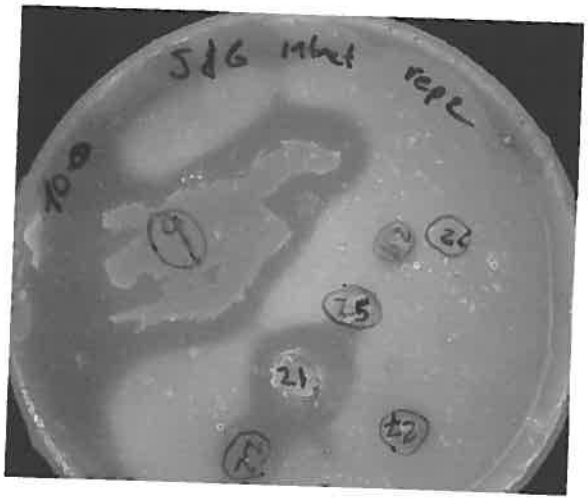

Figure 9. Development of an assay for the detection of the depletion of intact dDNA. A) control plate of intact dDNA stained with ethidium bromide; B) control plate with intact dDNA, a 25 μL drop of DNAse 1 in the middle, followed by ethidium bromide staining; C) bacterial isolate on intact dDNA plate showing negligible zones of DNA depletion (score 1); D) bacterial isolate on intact dDNA plate showing small zones of DNA depletion (score 2); E) bacterial isolate on intact dDNA plate showing medium zones of DNA depletion (score 3); F) bacterial isolate on intact dDNA plate showing large zones of DNA depletion (score 4).

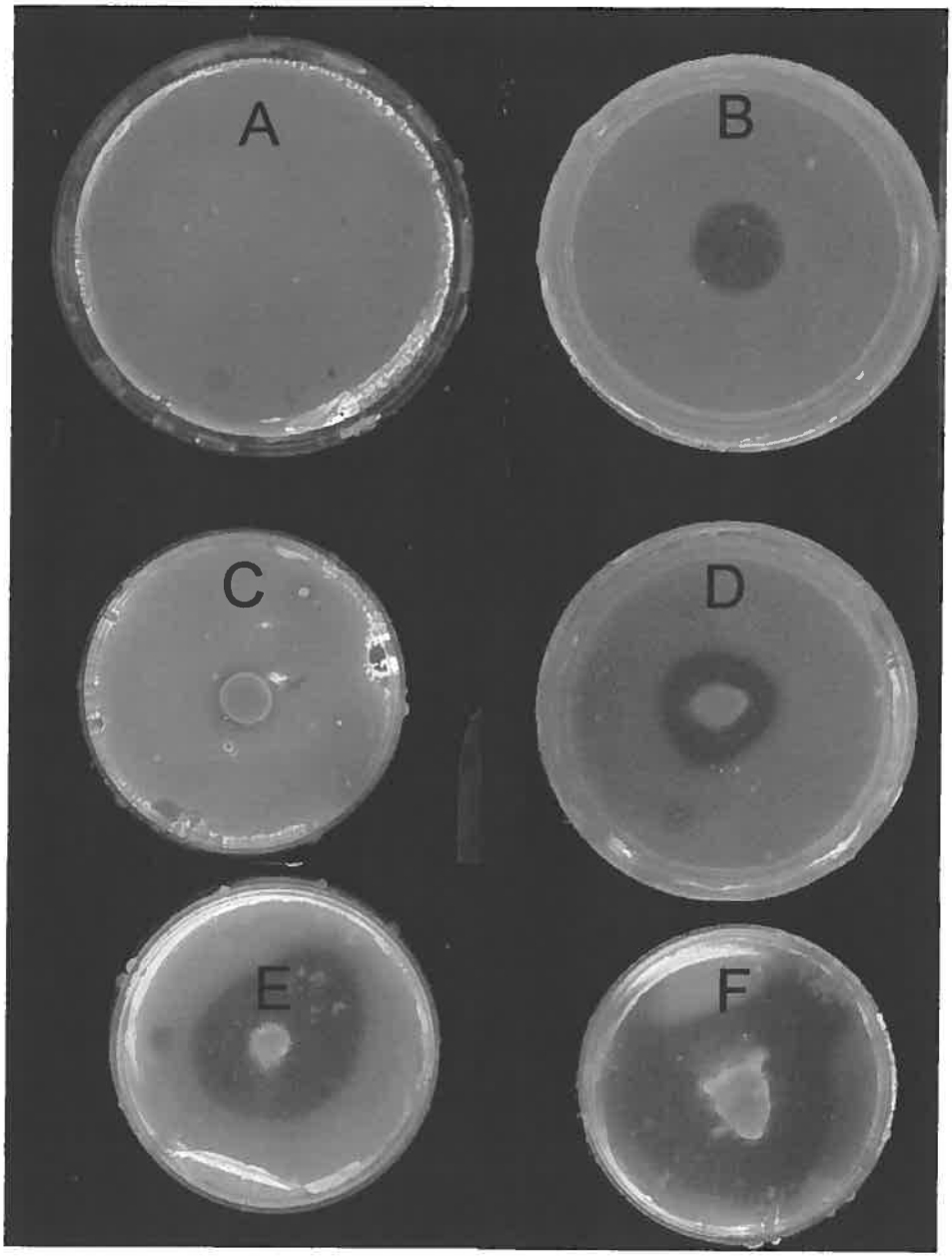

Figure 10. Proportion of dDA depletion from agar plates using the ethidium bromide depletion assay. Isolates from both intact and degraded DNA primary enrichments were transferred to new plates with intact dDNA. We qualitatively scored the zones of depletion as: 1 = 0-25%, 2 = 26-50%, 3 = 51–75%, 4 = 76-100%. Probability (*P*) value is a from a likelihood ratio generated from a contingency analysis.