Evaluation of Community-Level Physiological Profiling for Monitoring Microbial Community Function in Fish Hatchery Ponds

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ABSTRACT

Microbial communities of aquaculture ponds play pivotal roles in pond productivity and fish production success. Nonetheless, culturists do not consider this variable in pond management decision-making likely because of lack of practical assessment tools. Therefore, we evaluated the community-level physiological profiling (CLPP) technique for characterizing microbial community function for use in aquaculture. Un-filtered water samples collected from various plastic-lined ponds over time were incubated on Biolog™ EcoPlates, each with 3 replicates. Each replicate has 31 wells with 31 unique carbon substrates infused with a respiration-sensitive dye and a blank well (control). Responses (substrate optical densities) were measured at 24-h intervals for up to 12 d to determine the best incubation interval and required replicates for successful assessment of microbial community function in fish hatchery ponds. The repeatability and within-pond variability of the CLPP metrics also were evaluated. During cooler months and in ponds with no fish and low apparent microbial activity, a 96-h incubation period was required to differentiate microbial community functional characteristics. During warmer months and when ponds were stocked with fish and had significant phytoplankton blooms, incubation periods of 72 h or less were sufficient to distinguish communities. For routine monitoring, a single pond-water sample on one Biolog™ EcoPlate was adequate for detecting changes in microbial community function. Multiple water samples from a single pond revealed low heterogeneity in the microbial community function. When multiple Biolog™ EcoPlates were inoculated with a single pond-water sample, the dissimilarity of substrate responses was low (2.9%), indicating high repeatability of the CLPP technique. The CLPP method appears to be robust and enables assessment of heterotrophic microbial community functional characteristics such as relative diversity, similarity, and community functional activity. Therefore, it may offer the opportunity to assess hatchery pond microbial community function and lead to better understanding of the importance of the microbial community to the overall hatchery pond ecosystem function. Future studies should relate microbial community function to disease outbreaks, water quality variables, and zooplankton and phytoplankton population dynamics, and thereby develop a microbial community-based tool for pond management. Such a tool would allow improved and more comprehensive management of fish hatchery pond ecosystems.

INTRODUCTION

The structure and function of heterotrophic aquatic bacteria are important components of fish hatchery pond ecosystems (Wetzel 2001; Arias et al. 2006; Newton et al. 2011). These bacteria consume dissolved organic matter and nutrients for growth, mineralize organic matter to release nutrients to support productivity, serve as food sources to higher trophic-level organisms (Pace and Cole 1994; Fouilland and Mostajir 2010), and may cause or mediate fish diseases (Cunningham et al. 2012). Aquatic bacterial communities exhibit predictable patterns that are associated with seasonal phytoplankton and zooplankton blooms and water quality dynamics (Kent et al. 2007; Rösel and Grossart 2012). Microbial population density and activity are positively correlated with pond fertility and photosynthetic productivity, though the contributions of microbes to aquatic ecosystem metabolism can change rapidly (Xianzhen 1988; del Giorgio and Cole 1998; Wetzel 2001). Despite the apparent importance of the microbial community to pond production performance, practicing fish culturists do not routinely consider this variable in pond management decision-making because of lack of practical assessment tools.

This lack of tools is largely due to the assumption that the small size and high heterogeneity of microbes (Arias et al. 2006) make them more difficult to sample than plankton or fish. Further, there is the challenge to understanding microbial community function as opposed to identifying community composition, which is not unique to pond bacteria. The need to identify, enumerate, and understand complex aquatic phytoplankton communities led to the development of surrogate measures such as pond water color and turbidity, chlorophyll-*a* concentration, pH, and dissolved oxygen concentration. These metrics provide qualitative insights into the overall metabolic status of phytoplankton communities and are widely used in aquaculture where the quantitative specifics of community structure require specialized expertise (e.g., taxonomy) or a significant amount of time. Thus, development of a relatively fast and inexpensive tool to assess microbial community status, similar to the surrogate measures of phytoplankton communities, could be valuable to fish pond management. With such tools, aquaculturists could identify dense microbial populations when these dominate the pond ecosystem to the extent of potentially depleting dissolved oxygen or causing fish diseases and remove some by pond flushing. Further, the techniques could be used to positively influence microbial community function such as selectively enhancing a desirable microbial community to benefit pond fertility and productivity. With tools to assess and manage pond microbial communities, fertilizers might be more efficiently used in pond management, diseases better managed, and fish production enhanced. For example, Arias et al. (2006) used genetic techniques to monitor pond bacterial communities in Channel Catfish *Ictalurus punctatus* ponds and suggested that a collapse in bacterial species diversity might indicate impending environmental stress and disease. Therefore, they concluded that a better understanding of bacterial community function could lead to improved fish husbandry practices.

Genetic methods are difficult for most aquaculturists to employ on a production scale because of their complexity and cost. In addition, many of these methods determine microbial diversity, which may not be tightly coupled to microbial community function or functional diversity (Fisher et al. 2000; Mouchet et al. 2012). This is the case because a large portion of the microbial community may be dormant yet detectable with conventional counting or DNA extraction methods (Cole 1999). Conversely, the phenotypic technique of community-level physiological profiling (CLPP) with Biolog™ EcoPlates may offer the potential to assess gross

metabolisms of aquatic microbial communities in fish ponds, similar to the gross measures (e.g., dissolved oxygen concentration) for assessing phytoplankton community function. This technique has been widely used for soil matrices to confirm microbial community changes before and after some perturbation or to identify shifts in microbial community composition in space and time under varying environmental conditions (Garland 1997; Preston-Mafham et al. 2002; Insam and Goberna 2004). Schultz and Ducklow (2000) used CLPP to detect temporal and spatial changes in microbial communities along salinity gradients, and Sala et al. (2006) used it to determine the nitrogen-use status of aquatic microbial populations. Christian and Lind (2006) evaluated CLPP and concluded it provides valuable insight into bacterial substrate utilization and functional potential in aquatic ecosystems.

The CLPP technique measures the capacity of the heterotrophic microbial community to utilize select carbon substrates, and it involves incubating un-filtered water samples on commercially available Biolog™ EcoPlates (plates). Each plate contains triplicate micro wells of 31 unique carbon substrates and one well per replicate with no substrate to serve as a blank (Table 1). Microbial community function is revealed by analysis of the differential color development of formazan from the respiration-sensitive tetrazolium dye incorporated into each of the substrates (Weber and Legge 2006). Formazan is measurable as optical density (OD; absorbance at 590 nm). Each measurement of plate color development provides three community substrate utilization patterns (CSUPs), which can be evaluated at specific incubation time intervals or by analyzing the kinetics of color development and CSUP over time (Insam and Goberna 2004). The CSUP metrics quantify the overall rate of color development, richness and evenness of responses, and patterns of metabolism of the substrates which are functions of microbial community activity, density, diversity, and similarity (Garland 1997).

Haack et al. (1995) reported that the CLPP method is simple, relatively fast, and results are reproducible. For example, the method requires no sample preparation and enables detection of small shifts in microbial community function (Stefanowicz 2006). The method provides reliable assessment of bacterial community function because microbes have different preferences for substrates relative to their metabolic capabilities (Rösel and Grossart 2012). Because each plate contains a triplicate of the 32 set of wells, one water sample per pond per plate may be adequate for distinguishing among microbial communities from well-mixed ponds. Lowitt et al. (2000) reported that with one water sample from each of two well-mixed creeks incubated on one Biolog™ EcoPlate, CLPP distinguished between the microbial communities with a high degree of confidence (Power ≥ 0.95; $P = 0.05$). The CLPP method compares favorably with the genetic and biochemical techniques which are more complex and require specialized expertise for differentiating microbial communities (Najdegerami et al. 2012).

Nonetheless, the CLPP technique has limitations in its application to aquatic bacterial communities (Christian and Lind 2006). The color development of the formazan dye is temperature sensitive. Thus, samples must be incubated at a common temperature to overcome this limitation when comparing responses across wide environmental temperature ranges. Also, the method is quasi-culture dependent and could select for those heterotrophic bacteria (e.g., copiotrophic species) that grow best in the small (150-µL) plate wells with enriched carbon substrates (Smalla et al. 1998). If samples (e.g., especially soils) are not well-mixed, the influence of rare individuals or differences in inoculation densities may increase the variability of substrate responses and require a high number of replicates to improve statistical confidence

(Miguel et al. 2007). Data management associated with CLPP may be unwieldy for most aquaculturists. Each plate generates 96 OD values at each reading, resulting in a large dataset (potentially >1,000 data points for each sample over time). The CSUP data produce several metrics which are used to analyze and compare carbon substrate utilization by the heterotrophic microbial community (Garland et al. 2001). These include the kinetic parameters which describe the typical sigmoid response curve of OD values for each substrate (lag, slope, and maxima), the average of the OD values for all substrates at specific time intervals (average well color development of each plate replicate; AWCD), and the number of substrates that exhibit positive responses by developing color. Usually, a standard AWCD value in the middle of the exponential growth phase of the response curve is selected for comparison of communities (Schlutz and Ducklow 2000; Preston-Mafham 2002). Since data management is a potential issue with CLPP as a pond management tool, the efforts outlined herein focused on minimizing data collection and analysis to develop a practical tool for monitoring microbial community function in fish hatchery ponds.

The goal of this study was to evaluate some of the issues discussed above for future aquaculture applications and to determine if CLPP has the potential to acquire signals from the hatchery pond environment that can be useful for improving management. Our objectives were to 1) determine a suitable incubation time interval for reliable assessment of microbial community function for most fish hatchery ponds, 2) estimate the sampling effort (i.e. replicate water samples and replicate plates) required to distinguish between pond microbial communities, and 3) determine if CSUP metrics indicate significant differences in microbial community function (the degree of heterogeneity) over time and under differing aquaculture conditions.

MATERIALS AND METHODS

Biolog™ EcoPlates (Biolog, Inc., Hayward, California) were used for this study. Each plate consisted of three identical sections (replicates), each containing 31 micro wells with distinct dissolved organic carbon substrates and one well with no substrate to serve as negative control (blank; Table 1). Six ponds (survey ponds; < 0.5 ha each) at the Possum Kingdom State Fish Hatchery, Palo Pinto County, Texas were sampled up to seven times each from November 2009 through May 2010. A surface-water sample was collected from each pond on each sampling date at a depth of about 6 cm near the pond harvest structure. Water samples were collected in 50-mL sterile, plastic centrifuge tubes and immediately capped. All ponds were sampled in 30 min or less, and each sample was directly inoculated into the 96 micro wells (150 µl per well) of the plate within 30 min of collection (Lowit et al. 2000; Preston-Mafham et al. 2002) with an 8-channel pipettor. Plates where incubated at 21 ± 2°C in the dark (Christian and Lind 2006) for a maximum of 224 h or until plate AWCD exceeded OD of 1 (Weber and Legge 2010). Plates were read each day at 590 nm on a VersaMax™ microplate reader (Molecular Devices, Inc., Sunnyvale, California). Twenty-eight water samples were evaluated on 28 plates at multiple time intervals to produce 564 CSUPs.

Using these same methods, a single pond was sampled on 26 January 2010 from six different locations (two locations each at the front, middle, and back) to assess within-pond variability of CSUPs. Since there were triplicate wells on each plate, the sampling used 6 plates and yielded 18 CSUPs for this evaluation. We also evaluated within-sample variability of

CSUPs with water samples collected on 26 May 2010 and 01 June 2010 from two ponds each time (ponds 1 and 2, and 8 and 12, respectively). The water sample from each pond was inoculated on five plates, resulting in 15 replicates.

Individual plate readings were exported into Microsoft Excel™ for compilation and manipulation. Data from each plate were parsed into 3 data lines for the 3 replicates. For each plate reading data, the absorbance of each blank well was subtracted from the absorbance of each of the 31 wells of its plate replicate to calculate a net substrate absorbance value (Christian and Lind 2007). When blanking resulted in negative values, these values were set to zeros for data analysis.

For each replicate reading, AWCD was calculated as the sum of all blanked substrate absorbance values divided by 31. The number of positive substrates was calculated as the sum of all positive responses (up to 31). A substrate response was considered positive if its OD value after blanking was 0.2 or higher (Garland 1997). For analysis, each plate was treated as three individual samples albeit the same pond water sample was used to fill all wells of the plate (Christian and Lind 2006). The OD values of the three plate replicates were not averaged as is sometimes done to overcome potential variability in response due to low inoculum density or effects of rare individuals on responses (Preston-Mafham 2002). Because distribution of microbial cells in aquatic samples is typically more uniform than in other media (Konopka et al. 1997; Choi and Dobbs 1999) and our typical within-plate variation was low (discussed later), averaging replicate OD values which would have reduced the number of replicates and statistical power was unnecessary.

The AWCD, number of positive substrates, standard deviations (SD) of substrate OD, and number of substrate OD values exceeding 2 (substrate saturation; Weber and Legge 2010) were graphed over incubation time to determine the optimum plate incubation interval. Data from the survey ponds were combined and used to fit each of AWCD, number of positive substrates, and SD of substrate OD to a sigmoid curve with non-linear regression. A common sigmoid model was used to fit all response curves with JMP version 11 (SAS, SAS Campus drive, Cary, NC) by selecting the best-fit model that minimized the Akaike information criteria (AIC) and maximized *R*-square. This curve model was also used to fit curves for the single pond that was sampled 6 times from different locations as well as the four ponds that were each sampled twice and inoculated on to 5 EcoPlates each.

Multivariate procedures - analysis of similarity (ANOSIM), principle component analysis (PCA), and similarity percentage analysis (SIMPER) - were used to test for differences in CSUP metrics among pond water samples over incubation interval and to validate the choice of plate incubation interval with PAST version 2 (Hammer et al. 2001). For PCA, the variance-covariance matrix of OD values was used because all responses had a common scale of 0 to 3 (Hammer et al. 2001), and the first two principle component axes were used to evaluate differences of CSUPs. For ANOSIM, the Bray-Curtis index was used as the index of similarity (Bloom 1981). Analysis of variance (ANOVA) was used to compare AWCD and the number of positive substrates among ponds and within ponds over time with SYSTAT 13™ (SYSTAT Software, Inc., Chicago, Illinois). Where differences were significant among or within ponds, Bonferroni *t*-tests were performed to determine significant differences between means. Power analysis was performed to estimate the required sample size (number of plate replicates), given

the observed mean difference of AWCD and the number of positive substrates. Differences in CSUPs among ponds sampled at the same time or among ponds over time were evaluated with PCA and discriminant function analysis (DFA). Principle component analysis was used with no *a priori* group (pond) classification to determine if CSUPs yielded distinct clusters of pond samples when OD variance was reduced to the first two principle components. Conversely, DFA was used to evaluate the degree of separation between pond CSUPs with prior designation of groups and to evaluate those substrates that contributed the most to discrimination. For PCA and DFA, the covariance matrix was used, assuming similar variance and units for OD values. These analyses were performed with the Microsoft Excel™ add-in StatistiXL™ (http://www.statistixl.com, v1.10). For all analyses, statistical significance was set as $P \leq 0.05$.

RESULTS AND DISCUSSION

Incubation Interval

The AWCD data of the survey ponds followed the expected sigmoidal response with a lag phase occurring until about 50 h, an exponential growth phase between 50 and about 150 h, and a stationary phase beyond 150 h of the incubation period (Figure 1). The number of positive substrates and the SD of substrate OD values exhibited responses similar to that of the AWCD data (Figure 1 and 2). These three response curves were similar to those observed by Choi and Dobbs (1999) and Christian and Lind (2006) for freshwater environments. The AWCD, number of positive substrates, and SD of substrate OD data best fit (R-square ≥ 0.8451) a three-parameter sigmoidal Gompertz curve of the form:

$$x(h) = a * e^{-e^{-b*(h-c)}}$$

where h = incubation interval, a = curve asymptote (maximum AWCD, number of positives, or SD of substrate OD), b = curve shape parameter, c = curve inflection point (h), and e = 2.71828183 (the base of the natural logarithm). The parameters of the overall survey ponds AWCD curve were $a = 0.7792$, $b = 0.0230$ and $c = 78.7512$ h; the positive substrates curve were $a = 23.1295$, $b = 0.0318$ and $c = 63.8254$; and the SD of OD curve were $a = 0.6106$, $b = 0.0245$ and $c = 60.2171$ (Figures 1 and 2).

The appropriate incubation interval at which to compare CSUPs is where few substrate OD values exceed 2 (substrate saturation) and where the number of positive substrates and standard deviation of the sample OD are highest along the exponential growth phase of the sigmoid response curve (Weber and Legge 2010). This CSUP selection strategy maximizes the differences between individual plate CSUPs but retains the maximum number of wells within the exponential phase of the response curve. Based on these criteria, a maximum incubation interval for the water samples would not usually exceed 120 h because of the increase in OD values exceeded 2 (Figure 2). The inflection point of the AWCD curve occurred at about 79 h and the inflection point of the positive substrate curve occurred at about 64 h (Figure 1). A 72-h or 96-h incubation interval reduces the occurrence of OD values over 2 (Figure 2), retains a high number of positive substrates (Figure 1) and standard deviation (Figure 2), and provides a convenient 3- or 4-d incubation interval for aquaculture management activities.

The choice of optimal plate incubation time depends on preliminary examination of the OD data for the system under study (Weber and Legge 2010). Substrate OD values at a single point in time or characteristics of the sigmoid absorbance curve kinetics (area under the OD response curve) have been correlated with environmental variation of CLPP samples (Choi and Dobbs 1999). However, single absorbance point readings are recommended where the objective is to classify different microbial populations (Weber and Legge 2010). Further, Choi and Dobbs (1999) found that for aquatic samples a single incubation interval was as effective as the curve-kinetic approach. Therefore, we selected a time-point (based on the response curve parameters) as the primary basis for calculation of CSUP metrics because estimating curve kinetics is an additional complicated step that could be challenging to hatchery personnel. Using single time-point CSUP metrics, obtained at a short and common incubation interval, provides a manageable surveillance tool for hatchery managers who often manage large numbers of ponds and are challenged to make management decisions on many ponds quickly.

The AWCD averaged 0.23 (0.07 – 0.57) around the 72-h interval and 0.39 (0.07 - 0.96) around the 96-h interval (Figure 1). The latter value is within the range (0.25 - 1.0) recommended for microbial community classification (Garland 1997). Garland et al. (2001) suggested a minimum AWCD value of 0.25, but others have used higher minimum values (e.g., 0.5 by Schultz and Ducklow 2000). Based on these recommendations, it appears a minimum incubation time of 72 h may be adequate for hatchery pond microbial community function characterization. However, there is a risk that the lower variance of substrate OD might limit the ability to detect differences in microbial community function of ponds with the 72-h interval (Weber and Legge 2010). Substrate OD variance increased from 0.095 at 72 h to 0.176 at 96 h. Therefore, a 96-h interval might be required to improve detection of differences in microbial community function.

Based on the curve slopes during the exponential growth phase, the AWCD would increase by 0.1157 and the number of positive substrates would increase by 4.9231 for each 24-h increase in incubation time. The standard deviation of substrate OD values would increase by 0.1071 for each additional 24 h of incubation. Beyond 120 h, the number of positive substrates and the standard deviation of OD neared the asymptotes of the curves (Figure 1 and 2), indicating that incubation beyond 120 h would provide little new information. Schultz and Ducklow (2000) reported that maximum color development required 72 h in warmwater months and 240 h in cold-water months. We conducted this study during cooler and warm months (November-May; Figure 3) and the results agree with those of Schultz and Ducklow (2000). Our results suggest that an incubation interval greater than 72 h is required for cooler months whereas 72 h may be adequate for warmwater temperature periods with more microbial activity. However, future studies should verify this 72-h incubation interval for hatchery ponds during warmer months.

Because water samples were collected over a range of pond temperatures (Table 2) but incubated at a constant temperature, AWCD was modeled as a linear function of initial pond water temperature and incubation interval. The resulting regression was significant ($R^2 = 0.782$; $P = 0.000$) and yielded the relationship: AWCD = -0.082 + 0.004*h + 0.004* temperature (Figure 3). Solving for hours yielded a minimum incubation interval of about 72 h for an AWCD of 0.25 at most pond temperatures. A predefined incubation interval is more desirable than incubating samples to a target AWCD, which would require calculating AWCD over time.

Therefore, we recommend a minimum incubation interval of 72 h for the CLPP technique as long as pond temperatures exceed 10°C. A longer interval of 96 h might be necessary to differentiate microbial communities in colder water (temperature ≤10°C), but the additional 24-h incubation would reduce the potential to respond quickly to pond management issues, if the CLPP method were to be used as a tool. Where the monitoring period encompasses both warm and cold months, a 72-h incubation interval would reflect the inherently lower microbial activity associated with cold water temperatures and offer the capacity to distinguish these seasonal effects.

Principle component analysis, ANOSIM, and SIMPER provided further support for the 72- to 96-h plate incubation intervals for CLPP analysis (Figure 4). Dissimilarity of CSUPs decreased over time, but did not become more dissimilar beyond the 96-h incubation interval. The *R* statistic of ANOSIM peaked near the 72- to 96-h interval range and declined thereafter. This statistic was not significant for many of the comparisons prior to 72 h. The amount of variance explained by the first two principle components (and the ability to discriminate CSUPs) declined beyond the 96-h incubation period. The initial percentage of variance was high when few substrates had not developed full color because of the lag in substrate reduction. This scenario can lead to erroneous community classification with percentage variance (Weber and Legge 2010). Therefore, a minimum 72-h incubation period should reduce the risk of erroneous classification prior to the stabilization of substrate responses. This 72-h interval also minimizes incubation time because substrate responses beyond it usually do not provide significant additional information about community classification.

Within-Sample Variability of CSUPs

Ponds 1 and 2 were sampled on 26 May 2010 at a mean water temperature of 25 ± 1ºC. Pond 1 was least turbid with dissolved oxygen concentration of 7.1 mg/L. Pond 2 had a moderate phytoplankton bloom, dissolved oxygen concentration of 4.5 mg/L, and was stocked with Striped Bass *Morone saxatilis* fingerlings for about a month. On 1 June 2010, ponds 8 and 12, with water temperature of 28 ± 1ºC, were sampled. Pond 8 had a dense phytoplankton bloom and was stocked 3 months earlier with 450 kg of Koi Carp *Cyprinus carpio* fingerlings that had been heavily fed. The dissolved oxygen concentration in pond 8 was 4.5 mg/L at water sample collection. Pond 12 was fairly clear and had been filled and stocked about one week previously with Smallmouth Bass *Micropterus dolomieu* fingerlings. The dissolved oxygen in pond 12 was 6.7 mg/L at sampling. Based on these observations and assuming that microbial activity is correlated to apparent pond turbidity and dissolved oxygen (Xianzhen 1988; del Giorgio and Cole 1998; Wetzel 2001), we ranked microbial activity in the following decreasing order: pond 8 > pond 2 > pond 12 > pond 1.

The mean AWCD values at 72 h were 0.97, 0.34, 0.19, and 0.14 for ponds 8, 12, 1, and 2, respectively. The average number of positive substrates within each plate was correlated to AWCD and was 26, 12, 8, and 5 for ponds 8, 12, 1, and 2, respectively. For pond 8, one substrate well in about half of the 15 plate replicates exceeded OD of 2.0 (average = 0.47). None of the other plate substrates for the other ponds exceeded OD of 2. These results suggest, and thereby confirm, that the previously selected 72-h incubation interval was appropriate. However, the curve parameters of the four ponds (not shown) suggest that the longer incubation interval of 96 h might improve the ability to differentiate those ponds (1 and 2) that were similar in low substrate utilization. The estimated inflection points (*c*) of the AWCD curves were 40.9, 83.9,

110.2, and 87.3 h for ponds 8, 12, 1, and 2 respectively. This suggests that an additional 24 h of incubation may have been beneficial for distinguishing the two ponds of low apparent microbial activity. The corresponding AWCD curve shape parameters (*b*) were 0.0441, 0.0187, 0.0133, and 0.0153, respectively. The AWCD asymptotes were 1.26, 1.12, 1.04, and 0.4985, respectively. These parameters suggest an order of microbial activity and diversity in the following decreasing order: pond 8 > pond 12 > pond 1 > pond 2. This differed from the order suggested by observation alone and illustrates that CLPP may provide improved understanding of microbial function in aquaculture ponds.

When each of the 15 plate replicates inoculated from a single pond water sample was considered, the AWCD and number of positive substrates were significant for the pond effect ($P < 0.001$). For AWCD, ponds 1 and 2 were similar ($P = 0.142$), ponds 8 and 12 were dissimilar, and both were dissimilar from ponds 1 and 2 ($P < 0.001$). When OD values for each plate were averaged such that every plate was considered as an individual treatment ($N = 5$), multiple comparisons grouped AWCD and the number of positive substrates similar to the groupings provided by the combined 15 replicates per pond (data not shown). This result indicates a high level of repeatability of OD response among plates inoculated from the same pond water sample.

The variability of AWCD at 72 h was low within each pond water sample (Figure 5); standard deviations were 0.087, 0.072, 0.075, and 0.051 for ponds 8, 12, 1, and 2, respectively. The average standard deviation of AWCD for the four ponds' replicates was 0.071. Power analysis indicated that an AWCD difference of 0.2 between two ponds with three replicates would provide a power of 0.70% whereas 4 replicates would provide a power of 0.89%. An AWCD difference of 0.25 between ponds would provide a power of 89% with three replicates. Therefore, based on the observed standard deviations of the 4 pond samples and their 15 replicates each, a single plate with its three replicates should provide good power ($\geq 80\%$; Cohen 1988) to determine differences at a minimum mean difference in AWCD of 0.25 between ponds. Smaller differences in AWCD might require 2 plates (6 replicates) to achieve adequate power to determine differences between ponds.

The number of positive substrates was dissimilar among all 4 ponds (maximum $P = 0.001$), and the standard deviations were 1.5, 3.3, 4.5, and 2.0 for ponds 8, 12, 1, and 2, respectively (Figure 5). Power analysis indicated that detecting a significant difference of 5 positive substrates between two ponds required 6 replicates (two plates) per pond. Detecting a difference of 10 positive substrates would provide a power of 89.3% with 3 replicates (one plate). One plate at the observed standard deviation would provide 80% power to detect a minimum difference of 9 positive substrates between two ponds. Therefore, if only one plate were used for routine monitoring of ponds, positive substrate utilization differences of less than 9 substrates would have to be interpreted with caution.

Principle component analysis of CSUP (or OD values) visually confirmed results similar to those obtained by ANOVA of AWCD and number of positive substrates (Figure 6). A high amount of the variance (81%) in CSUP (or OD values) was explained by two principle components and 95% confidence intervals, and the 15 individual replicates indicated consistent ordination of pond EcoPlate responses (Figure 6). Ponds 8 and 12 appeared to be distinct in CSUP from ponds 1 and 2 whereas ponds 1 and 2 appeared to have similar CSUPs. Repeated principle component analysis with data from each of the 5 plates individually yielded a similar

ability to distinguish ponds in a similar fashion with the variance explained by the first two principle components ranging from 81 to 88%, indicating a high degree of precision with a single EcoPlate and its three replicates.

Analysis of similarity of substrate OD further confirmed significant differences among all four ponds ($P = 0.0006$). Dissimilarity was greatest between ponds 8 and 12 ($R = 0.9917$), and pond 8 was also highly dissimilar from pond 1 ($R = 0.8474$) and pond 2 ($R = 0.9834$). Pond 12 was also dissimilar from pond 1 ($R = 0.5604$) and pond 2 ($R = 0.722$). Although ponds 1 and 2 were significantly different, the R value of 0.2616 was low. Overall dissimilarity (SIMPER = 59.6) was greatest for the OD values of pond 8 versus ponds 1 and 2 (SIMPER = 69.7 and 75.6), respectively. Carbohydrate substrates accounted for most of the overall average dissimilarity.

Within-Pond Variability of CSUPs

The water in pond 1 was relatively clear at sample collection, suggesting low phytoplankton density and low overall microbial diversity and density (Worm et al. 2001). Pond water was fairly cool (9.5°C), and the apparent microbial community function was low. Few substrates (D-Galacturonic acid, L-Asparagine, and D-Mannitol) exceeded the saturation OD of 2 by the end of plate incubation (196 h). The OD response curves plateaued at an average curve asymptote of 0.779 AWCD and 21.9 positive substrates (Figure 7). The 72-h incubation interval preceded the average curve AWCD inflection point of 92.5 h (Figure 7). The inflection points for positives substrates and SD of OD responses, however, were 70.3 h and 73.5 h, respectively. We were unable to collect OD measurements at the 96-h interval and therefore examined the 120-h interval which still preceded the average AWCD curve asymptote.

The AWCD averaged 0.17 (0.11 - 0.27) at 72 h and 0.41 (0.28 - 0.55) at 120 h. Thus, the longer 120-h incubation interval provided an average AWCD closer to the recommended minimum of 0.25 (Garland et al. 2001) or 0.5 (Schultz and Ducklow 2000). The individual AWCD response curve inflection points ranged from 86 to 107 h, further supporting this choice of 120 h interval. The range of AWCD at 120 h (0.2622) was less than the AWCD of 0.3 which provided 95.3% power to detect differences among AWCD of four individual ponds, suggesting that the samples at all 6 pond locations were similar in terms of AWCD. At the 120-h interval, the SD of all 18 replicates was 0.07739, 3.2079, and 0.0023 for AWCD, number of positive substrates, and SD of substrate OD, respectively. The coefficient of variation for the AWCD, number of positive substrates, and SD of substrate OD was 18.9%, 17.8%, and 13.4%, respectively. The sample variances were similar for AWCD ($P = 0.8683$), positive substrates ($P = 0.9540$), and SD of substrate OD ($P = 0.9769$). This low variation in responses suggests that a single water sample (with its three replicates) can be used to determine overall microbial activity of an individual pond.

The SIMPER dissimilarity index was very low for both 72- and 120-h incubation intervals (2.9% and 3.4%, respectively), and the R values of ANOSIM indicated very low ability to separate communities at both 72 and 120 h (0.16 and 0.31, respectively). A PCA plot of CSUPs at 120 h revealed similarities, as indicated by poor separation by location among the samples and low total variance of the two first principle components (35.9%; Figure 8). The 95% confidence intervals of individual samples were beyond the range illustrated in Figure 8, unlike the confidence interval observed for the four individual ponds (Figure 6).

In spite of the apparent similarities in EcoPlate responses found within samples from a single pond, responses of four of the substrates - D, L-α-glycerol phosphate ($P = 0.0283$), D-galactonic acid γ-lactone ($P = 0.0232$), L-Arginine ($P = 0.0002$), and 4-hydroxy benzoic acid ($P = 0.0103$) - differed significantly among the 6 samples. Therefore, the issue of sampling precision and repeatability should be further explored in ponds with higher apparent microbial community function.

These results suggest a microbial functional homogeneity within a pond, at least at this apparent low microbial density. This observation differs from CLPP analysis of soil samples where low microbial density yields more variable CSUP because of the over-weighted influence of rare community members, requiring more replicates to improve statistical confidence (Miguel et al. 2007). The higher miscibility of water compared to soil media may explain the high homogeneity of the microbial community revealed in this study. The water samples for this study did not appear to contain large particulate matter that might be unevenly dispersed among individual wells of a plate. Even if this were a concern, Worm et al. (2001) found functional similarity of attached and free-living bacteria in freshwater plankton blooms. Therefore, these results suggest that a single pond-water sample incubated on one Biolog™ EcoPlate with its three replicate set of wells, provides adequate assessment of microbial community function within a single pond.

Temporal Variability of CSUPs among Ponds

Number of positive substrates and AWCD significantly differed among ponds on individual sampling dates and within ponds over time (Table 3; Figure 9). Analysis of variance revealed these differences despite AWCD differences of less than the suggested threshold of 0.3 provided by power analysis and the 10 positive substrates threshold of power analysis for one plate. The number of positives substrates and AWCD were highest for ponds characterized as "green" in color and had received high feed inputs from feeding of stocked Channel Catfish or Koi Carp. This apparent enhancement of bacterial community function in phytoplankton-, nutrient-, and organic matter-rich aquatic environments is consistent with previous findings (del Giorgio and Cole 1998).

High AWCD may result from a few substrates responding strongly and from many substrates responding moderately to bacterial metabolism due to high microbial biomass and higher community diversity, respectively, or a combination of both factors. However, the number of positive substrates usually increases with high AWCD, and it is often difficult to distinguish with certainty whether community biomass or community diversity differentiates communities. In the case of the greatest difference observed between ponds in this survey (ponds 15 vs. 44 on 9 March 2010; Table 3), it appears both biomass and diversity were highest in pond 15 at a time when water quality characteristics were not apparently different. Pond 15 had 13 more positive substrates than pond 44 and the average proportion of AWCD per substrate was higher (AWCD/number of positives = 0.032 vs. 0.020), indicating that a greater diversity of substrates were more highly metabolized by the microbial community of pond 15.

Discriminant analysis of CSUPs allowed further exploration of the differences among pond microbial communities based on the particular substrates that responded to microbial metabolism. Eight substrates discriminated 99.6% of the variability in CSUPs among ponds on 9 March 2010 (Figure 10). Pond 15 was easily distinguished by higher utilization of 6 substrates

comprised of two carbohydrates (D, L-α-glycerol phosphate, and glucose-1-phosphate), three carboxylic acids (α-Ketobutyric acid, D-Glucosaminic acid, and D-Malic acid), and one amine (Putrescine). Even on 20 January 2010, when the number of positive substrates and AWCDs where indistinguishable by ANOVA (Table 3), discriminate analysis distinguished ponds by CSUP (Figure 11). A similar discriminatory ability of CSUP was observed when individual ponds were examined over time. Pond 19 appeared to diverge in substrate-use during the warmer months and was characterized by increased use of 10 substrates (Figure 12), especially on 9 March 2010 which was when the highest AWCD and positive substrates of all sampling dates were observed.

A closer examination of CSUPs and hypothesis about changes in specific substrate utilization associated with microbial community composition or other pond characteristics were beyond the scope of this study. However, the responses observed in this survey appear consistent with reports that microbial population density and metabolism are positively correlated with pond fertility and photosynthetic productivity (Xianzhen 1988; Wetzel 2001).

Conclusions

The purpose of this research was to estimate a suitable Biolog™ EcoPlate incubation interval and required sampling effort for successful assessment of microbial community function, and determine if CLPP revealed differences in heterotrophic microbial community function in hatchery ponds under differing aquaculture conditions. We found a single pond water sample incubated for 72 h adequate for characterizing pond water microbial community function during November-May in Texas. Thus, large numbers of ponds can be sampled in a short time during this period. Significant differences in CSUP metrics between ponds and within ponds over time were detected, indicating adequate sensitivity of the CLPP method in discriminating between microbial community functional characteristics. These results should allow design of experiments with many pond replicates or regular monitoring of many ponds during routine aquaculture. Generally, AWCD and the number of positive substrates provided discrimination among ponds, similar to estimating curve parameters, provided the appropriate incubation interval in the 72- to 120-h range was used. These outcomes satisfy our goal of minimizing data collection in order to develop a practical tool for monitoring microbial community function in fish hatchery ponds. Future studies should verify if the 72-h incubation interval is also appropriate for June-October.

The best choice of incubation interval will likely be a function of the time frame of the study and the ambient microbial productivity of the aquaculture system being studied. Therefore, although we provide some guidance herein, we recommend that incubation curves be examined within the system under study to validate this “best interval” prior to study implementation. To simplify this task, numerical curve fitting may be unnecessary and these curves may be examined graphically to estimate the time period between the end of the lag phase and prior to the curve asymptote. The area between these two curve points (72- to 120-h) corresponded to the exponential growth phase of OD values in our ponds where AWCD and the number of positive substrates can be used for comparison purposes.

We used several statistical procedures to analyze various CSUP metrics in this study. These procedures provided similar results, but were necessary to examine the complexity of EcoPlate data and confirm the simplest analysis. However, analysis of variance and comparison

of means of AWCD and the number of positive substrates from EcoPlate OD data may be adequate to detect differences between ponds where differences are significant from a pond management perspective. It is conceivable then, that with further development, pond water samples could be collected on Monday of a typical work week and results could be available to inform management decisions by Friday. Analysis and comparison of OD response curve characteristics and the responses of individual substrates offer the potential to detect subtler or more specific differences in pond microbial function, if necessary.

To achieve the ultimate goal of developing a practical tool for assessing microbial community function for the purpose of fish pond productivity management would require additional studies. The CLPP technique should be used to explore some of the fundamental questions and assumptions about the microbial community of aquaculture ponds and to determine if the assay is specific and sensitive enough to detect changes in microbial community function that are related to pond management issues. A few of these issues or questions are: Do changes in microbial community function precede fish disease outbreaks or episodes of poor water quality; and if so, can CLPP be used to predict and interdict these problems? What pond management activities alter the function of aquatic microbial communities, and do these changes significantly impact fish production? For example, do chemical treatments alter the microbial community function of aquaculture ponds; and if so, what is the consequence to fish production? Answers to such questions would contribute to improving our understanding of the microbial community function, as a component of the pond productivity machinery, and ultimately result in the development of a tool that would contribute to more comprehensive and effective management strategies for fish hatchery ponds.

LITERATURE CITED

Arias, C. R., J. W. Abernathy, and Z. Liu. 2006. Combined use of 16S ribosomal DNA and automated ribosomal intergenic space analysis to study the bacterial community in catfish ponds. Letters in Applied Microbiology 43:287-292.

Bloom, S. A. 1981. Similarity indices in community studies: potential pitfalls. Marine Ecology Progress Series 5:125-128.

Boyd, C. E. 1990. Water quality in ponds for aquaculture. Alabama Agricultural Experiment Station, Auburn University, Auburn.

Choi, K., and F. C. Dobbs. 1999. Comparison of two kinds of Biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. Journal of Microbiological Methods 36:203-213.

Christian, B. W., and O. T. Lind. 2006. Key issues concerning Biolog use for aerobic and anaerobic freshwater bacterial community-level physiological profiling. International Review of Hydrobiology 91:257-268.

Christian, B. W., and O. T. Lind. 2007. Multiple carbon substrate utilization by bacteria at the sediment-water interface: seasonal patterns in a stratified eutrophic reservoir. Hydrobiologia 586:43-56.

Cohen, J. 1992. A power primer. Psychological Bulletin 112(1): 155-159.

Cunningham, F. L., S. W. Jack, D. Hardin, and R. W. Willis. 2012. Pond-level risk factors associated with Columnaris disease on Mississippi commercial catfish farms. Journal of Aquatic Animal Health 24(3):178-184.

del Giorgio, P. A., and J. J. Cole. 1998. Bacterial growth efficiency in natural aquatic systems. Annual Review of Ecology and Systematics 29:503-541.

Docherty, K. M., K. C. Young, P. A. Maurice, and S. D. Bridgham. 2006. Dissolved organic matter concentration and quality influences upon structure and function of freshwater microbial communities. Microbial Ecology 52(3):378-388.

Fisher, M. M., J. L. Klug, G. Lauster, M. Newton, and E. W. Triplett. 2000. Effects of resources and trophic interactions on freshwater bacterioplankton diversity. Microbial Ecology 40:125-138.

Fouilland, E., and B. Mostajir. 2010. Revisited phytoplankton carbon dependency of heterotrophic bacteria in freshwaters, transitional, coastal and oceanic waters. FEMS Microbiological Ecology 73:419-429.

Garland, J. L., and A. L. Mills. 1991. Classification and characterization of heterotrophic microbial communities on the basis of community-level-sole-carbon-source-utilization. Applied Environmental Microbiology 57:2351-2359.

Garland, J. L. 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology. Federation of European Microbiological Societies Microbiology Ecology 24:289-300.

Garland, J. L., A. L. Mills, and J. S. Young. 2001. Relative effectiveness of kinetic analysis vs. single point readings for classifying environmental samples based on community-level physiological profiles (CLPP). Soil Biology and Biochemistry 33:1059-1066.

Griffin, M. J., M. J. Mauel, T. E. Greenway, L. H. Khoo, and D. J. Wise. 2011. A real-time polymerase chain reaction assay for quantification of *Edwardsiella ictaluri* in catfish pond water and genetic homogeneity of diagnostic case isolates from Mississippi. Journal of Aquatic Animal Health 23:178-188.

Haack, S. K., H. Garchow, M. J. Klug, and L. J. Forney. 1995. Analysis of factors affecting accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. Applied Environmental Microbiology 61(4):1458-1468.

Hammer, Ø., D. A. T. Harper, and P. D. Ryan. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4(1):9 pp.

Insam, H., and M. Goberna. 2004. Use of Biolog® for the Community Level Physiological Profiling (CLPP) of environmental samples. Molecular Microbial Ecology Manual, Second Edition 4.01:853-860. Kluwer Academic Publishers, the Netherlands.

Kent, A. D., A. C. Yannarell, J. A. Rusak, E. W. Triplett, and K. D. McMahon. 2007. Synchrony in aquatic microbial community dynamics. International Society for Microbial Ecology 1:38-47.

Konopka, A., L. Oliver, and R. F. Turco, Jr. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. Microbial Ecology 35:103-115.

Lehman, R. M., F. S. Colwell, and J. L. Garland. 1997. Physiological profiling of indigenous aquatic microbial communities to determine toxic effects of metals. Environmental Toxicology and Chemistry 16(11):2232-2241.

Lyon, M. M., and F. C. Dobbs. 2012. Differential utilization of carbon substrates by aggregate-associated and water-associated heterotrophic bacterial communities. Hydrobiologia 686(1):181-193.

Mouchet, M. A., C. Bouvier, T. Bouvier, M. Troussellier, A. Escalas, and D. Mouillot. 2012. Genetic difference but functional similarity among fish gut bacterial communities through molecular and biochemical fingerprints. FEMS Microbiological Ecology 79:568-580.

Najdegerami, E. H., T. N. Tran, T. Defoirdt, M. Marzorati, P. Sorgeloos, N. Boon, and P. Bossier. 2012. Effects of poly-β-hydroxybutyrate (PHB) on Siberian sturgeon (*Acipenser baerii*) fingerlings and its gastrointestinal tract microbial community. FEMS Microbiology Ecology 79:25-33.

Newton, R. J., S. E. Jones, A. Eiler, K. D. McMahon, and S. Bertilsson. 2011. A guide to the natural history of freshwater lake bacteria. Microbiology and Molecular Biology Reviews 75(1):14-49.

Pace, M. L, and J. J. Cole. 1994. Comparative and experimental approaches to top-down and bottom–up regulation of bacteria. Microbiological Ecology 28:181-193.

Preston-Mafham, J., L. Boddy, and P. F. Randerson. 2002. Analysis of microbial community functional diversity using sole-carbon-source utilization profiles – a critique. FEMS Microbiology Ecology 42:1-14.

Ramette, A. 2007. Multivariate analyses in microbial ecology. FEMS Microbiological Ecology 62:142-160.

Rösel, S., and H. P. Grossart. 2012. Contrasting dynamics in activity and community composition of free-living and particle-associated bacteria in spring. Aquatic Microbial Ecology 66:169-181.

Sala, M. M., J. Pinhassis, and J. M. Gasol. 2006. Estimation of bacterial use of dissolved organic nitrogen compounds in aquatic ecosystems using Biolog plates. Aquatic Microbial Ecology 42:1-5.

San Miguel, C., M. Dulinski, and R. L. Tate, III. 2007. Direct comparison of individual substrate utilization from a CLPP study: A new analysis for metabolic diversity data. Soil Biology and Biochemistry 39:1870-1877.

Schultz, Jr., G. E., and H. Ducklow. 2000. Changes in bacterioplankton metabolic capabilities along a salinity gradient in the York River estuary, Virginia, USA. Aquatic Microbial Ecology 22:163-174.

Sinsabaugh, R. L., and C. M. Foreman. 2001. Activity profiles of bacterioplankton in a eutrophic river. Freshwater Biology 46:1239-1249.

Stefanowitcz, A. 2006. The Biolog plates technique as a tool in ecological studies of microbial communities. Polish Journal of Environmental Studies 15(5):669-676.

Van der Merwe, T., F. Wolfaardt, and K. Riedel. 2003. Analysis of the functional diversity of the microbial communities in a paper-mill water system. Water SA 29(1):31-34.

Weber, K., and R. Legge. 2010. Community-level physiological profiling. Pages 263-281 *in* S. Cummings, editor. Bioremediation: methods and protocols. Humana Press, New York, New York.

Wetzel, R. G. 2001. Limnology: Lake and River Ecosystems. Third edition. Elsevier Academic Press, San Diego, California.

Worm, J., K. Gustavson, K. Garde, N. H. Borck, and M. Søndergaard. 2001. Functional similarity of attached and free-living bacteria during freshwater phytoplankton blooms. Aquatic Microbial Ecology 25:103-111.

Xianzhen, G., F. Yingxue, W. Jikun, and P. Xiuzhen. 1988. Preliminary studies on the analysis of bacterial types in the fish ponds applied with four kind of animal manure and the effects of manuring on the ecosystem and yield. Network of Aquaculture Centres in Asia, National Inland Fisheries Institute, Kasetsart University, Bangkok, Thailand. www.fao.org/docrep/field/009/ag149e/AG149E01.htm *accessed 04/28/2010.*

Zinger, L., A. Gobett, and T. Pommier. 2012. Two decades of describing the unseen majority of aquatic microbial diversity. Molecular Ecology 21:1878-1896.

TABLE 1.—Carbon substrates of the Biolog™ EcoPlate wells. Each Biolog™ EcoPlate contains 96 wells consisting of three replicates (first three columns) of 31 substrates and 1 water blank.

96 EcoPlate cell labels			Substrate
A1	A5	A9	None (water blank)
B1	B5	B9	Pyruvic acid methyl ester
C1	C5	C9	Tween 40
D1	D5	D9	Tween 80
E1	E5	E9	α-Cyclodextrin
F1	F5	F9	Glycogen
G1	G5	G9	D-Cellobiose
H1	H5	H9	α-D-Lactose
A2	A6	A10	β-Methyl-D-glucoside
B2	B6	B10	D-Xylose
C2	C6	C10	I-Erythritol
D2	D6	D10	D-Mannitol
E2	E6	E10	N-Acetyl-D-glucosamine
F2	F6	F10	D-Glucosaminic acid
G2	G6	G10	Glucose-1-phosphate
H2	H6	H10	D,L-α-Glycerol phosphate
A3	A7	A11	D-Galactonic acid γ-Lactone
B3	B7	B11	D-Galacturonic acid
C3	C7	C11	2-Hydroxy benzoic acid
D3	D7	D11	4-Hydroxy benzoic acid
E3	E7	E11	γ-Hydroxybutyric acid
F3	F7	F11	Itaconic acid
G3	G7	G11	α-Ketobutyric acid
H3	H7	H11	D-Malic acid
A4	A8	A12	L-Arginine
B4	B8	B12	L-Asparagine
C4	C8	C12	L-Phenylalanine
D4	D8	D12	L-Serine
E4	E8	E12	L-Threonine
F4	F8	F12	Glycyl-L-glutamic acid
G4	G8	G12	Phenylethylamine
H4	H8	H12	Putrescine

TABLE 2.—Characteristics of ponds from which water samples were collected for community-level physiological profiling. Date is date of sample collection. XXX is no fish, CCF is Channel Catfish fingerlings, KOI is Common Carp fingerlings, and SMB is Smallmouth Bass brood fish.

Observation	Pond 1	Pond 13	Pond 15	Pond 19	Pond 21	Pond 44
			11/06/2009			
Water color	clear	.	.	clear	green	.
Fish	XXX	.	.	CCF	KOI	.
pH	8.3	.	.	8.6	9.2	.
Temperature (°C)	17.6	.	.	17.1	16.3	.
Dissolved oxygen (mg/L)	9.7	.	.	9.3	9.4	.
			11/16/2009			
Water color	clear	.	.	clear	green	.
Fish	XXX	.	.	CCF	KOI	.
pH	8.1	.	.	8.4	8.8	.
Temperature (°C)	17.2	.	.	15.9	12.2	.
Dissolved oxygen (mg/L)	9.4	.	.	9.4	9.5	.
			11/30/2009			
Water color	clear	clear	.	clear	.	green
Fish	XXX	CCF	.	CCF	.	SMB
pH	8.4	9.4	.	8.4	.	8.4
Temperature (°C)	13.4	11.9	.	9.6	.	10.2
Dissolved oxygen (mg/L)	10.6	10.6	.	11.5	.	11.2
			12/28/2009			
Water color	clear	clear	.	clear	.	green
Fish	XXX	CCF	.	CCF	.	SMB
pH	8.3	8.4	.	8.3	.	8.8
Temperature (°C)	4.7	3.8	.	4.1	.	4.6
Dissolved oxygen (mg/L)	10.9	11.0	.	11.4	.	11.0
			01/20/2010			
Water color	clear	clear	green	clear	.	green
Fish	XXX	CCF	CCF	CCF	.	SMB
pH	8.4	8.4	8.5	8.4	.	8.9
Temperature (°C)	11.5	10.6	11.1	10.8	.	10.7
Dissolved oxygen (mg/L)	11.9	11.6	11.8	11.9	.	12.1
			03/09/2010			
Water color	clear	clear	green	clear	.	green
Fish	XXX	CCF	CCF	CCF	.	SMB
pH	8.3	8.0	8.5	8.1	.	7.8
Temperature (°C)	13.2	13.2	13.2	13.1	.	12.9
Dissolved oxygen (mg/L)	10.3	11.2	9.2	10.1	.	8.8
			05/03/2010			
Water color	clear	green	.	green	green	.
Fish	XXX	CCF	.	CCF	KOI	.
pH	8.1	9.1	.	8.8	9.2	.
Temperature (°C)	17.7	20.8	.	18.9	21.2	.
Dissolved oxygen (mg/L)	10.6	8.9	.	10.2	9.0	.

TABLE 3.—Results of analysis of variance and means comparisons of average well color development (AWCD) and number of positive substrates (Positives) for ponds from which water samples were collected for community-level physiological profiling with Biolog EcoPlates™. Date is date of sample collection. Values bearing identical superscripts (a, b, or c) in the same row and values with identical superscripts (w, x, y, or z) in the same column are not significantly different ($P > 0.05$). Bottom two rows are P values for individual ponds over time.

	Pond						
Variables	1	13	15	19	21	44	***P***
				11/6/2009			
AWCD	0.22 ^{a,x,y}	.	.	0.16 ^{a,w}	0.32 ^{a}	.	0.065
Positives	8.0 ^{a,x,y}	.	.	8.7 ^{a,w,x}	13.3	.	0.004
				11/16/2009			
AWCD	0.22 ^{a,x,y}	.	.	0.23 ^{a,w}	0.37 ^{a}	.	0.067
Positives	11.7 ^{a,b,x,y}	.	.	9.3 ^{b,w,x}	14.7 ^{a}	.	0.032
				11/30/2009			
AWCD	0.21 ^{a,x,y}	0.09 ^{b,w}	.	0.13 ^{a,b,w}	.	0.15 ^{a,b,w}	0.009
Positives	10.7 ^{a,x,y}	4.7 ^{a,w}	.	6.7 ^{a,x}	.	5.3 ^{a,w}	0.035
				12/28/2009			
AWCD	0.21 ^{a,b,x,y}	0.29 ^{a,x,y}	.	0.15 ^{b,w}	.	0.14 ^{b,w}	0.001
Positives	10.3 ^{a,b,x,y}	13.3 ^{b,x,y}	.	9.7 ^{a,b,w,x}	.	6.3 ^{a,w}	0.003
				1/20/2010			
AWCD	0.18 ^{a,y}	0.20 ^{a,w,x}	0.24 ^{a}	0.23 ^{a,w}	.	0.25 ^{a,w}	0.625
Positives	10.3 ^{a,y}	8.3 ^{a,w,x}	12.0 ^{a}	13.3 ^{a,w}	.	11.0 ^{a,w,x}	0.382
				3/9/2010			
AWCD	0.31 ^{a,x}	0.43 ^{a,y,z}	0.87	0.43 ^{a}	.	0.28 ^{a,w}	0.000
Positives	16.3 ^{a,b,x}	19.0 ^{a,z}	27.0	20.0 ^{a}	.	13.7 ^{b,x}	0.000
				5/3/2010			
AWCD	0.12 ^{a,y}	0.50 ^{b,z}	.	0.23 ^{a,w}	0.47 ^{b}	.	0.000
Positives	6.3 ^{a,y}	18.0 ^{b,y,z}	.	9.7 ^{a,c,w,x}	16.7 ^{b,c}	.	0.003
AWCD	0.005	0.000	0.001	0.000	0.104	0.042	-
Positives	0.007	0.000	0.002	0.000	0.115	0.010	-

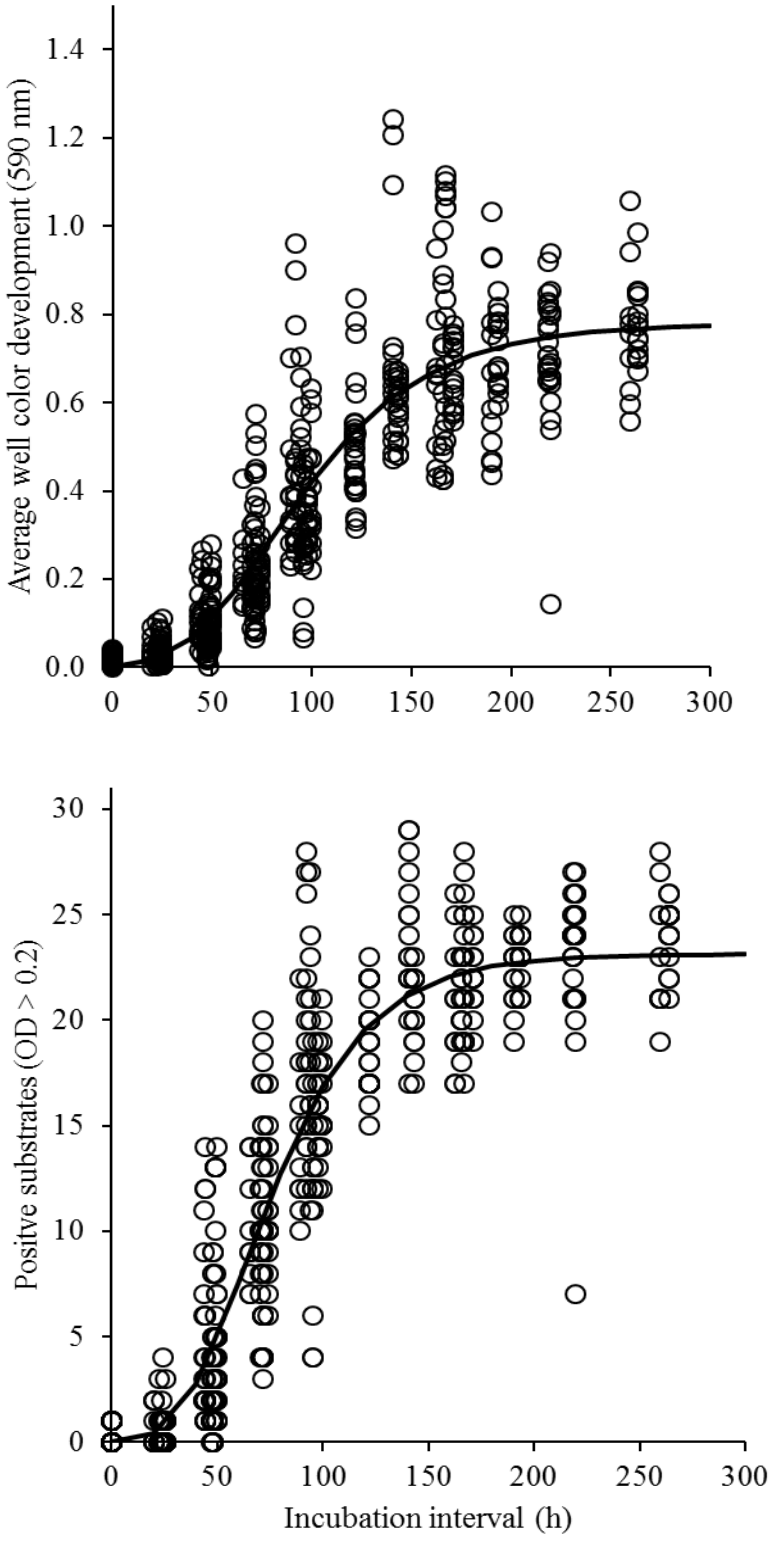

FIGURE 1.—Average well color development values and numbers of positive Biolog™ EcoPlate substrates at increasing incubation intervals for 28 water samples from Possum Kingdom State Fish Hatchery ponds in November 2009 - May 2010. The solid trend line is a best-fit sigmoid curve.

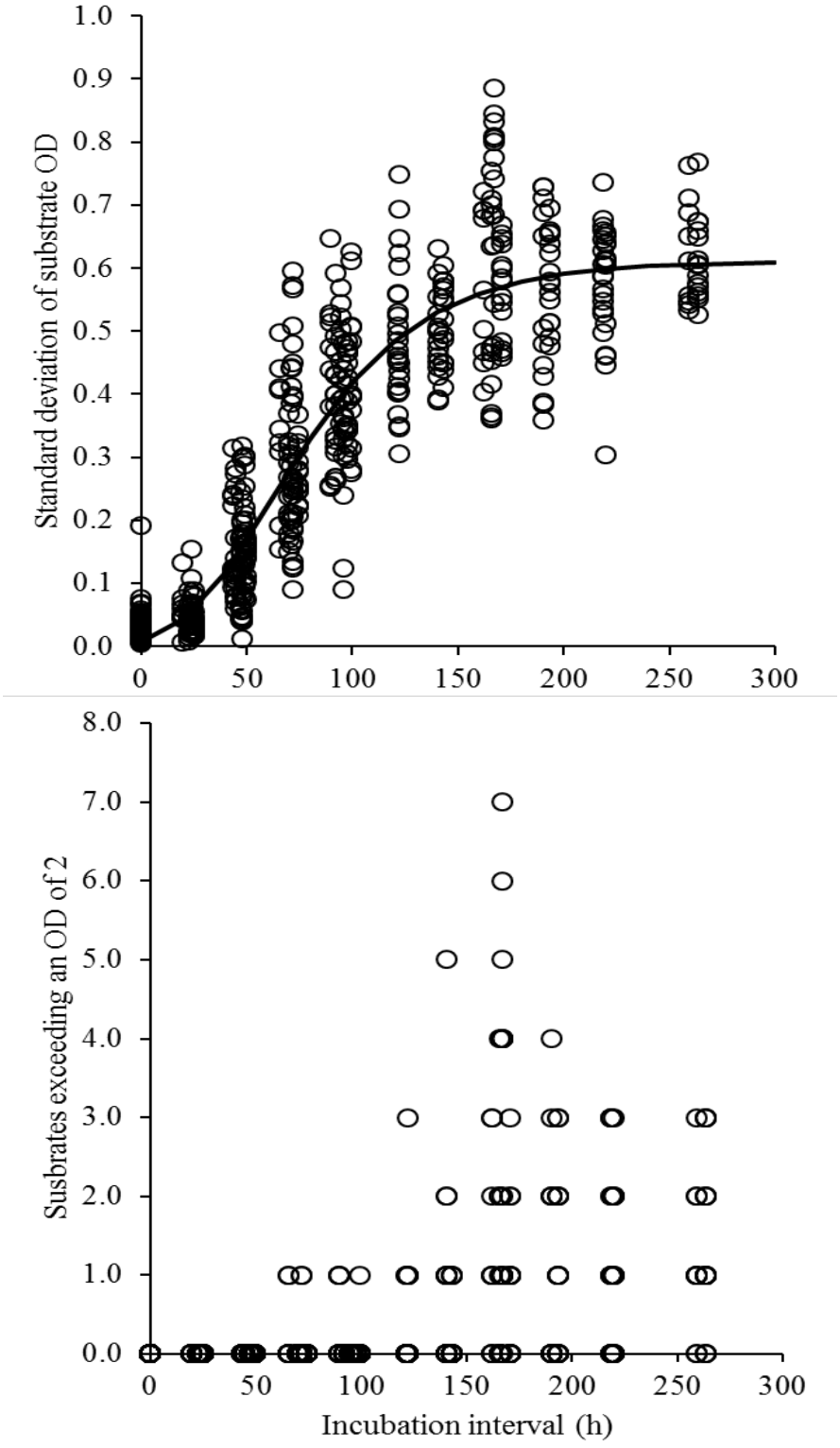

FIGURE 2.—Standard deviations of Biolog ™ EcoPlate substrate optical densities (OD) at 590 nm and numbers of substrates with OD > 2 at increasing incubation intervals for 28 water samples collected from Possum Kingdom State Fish Hatchery ponds during November 2009 - May 2010. The solid trend line is a best-fit sigmoid curve.

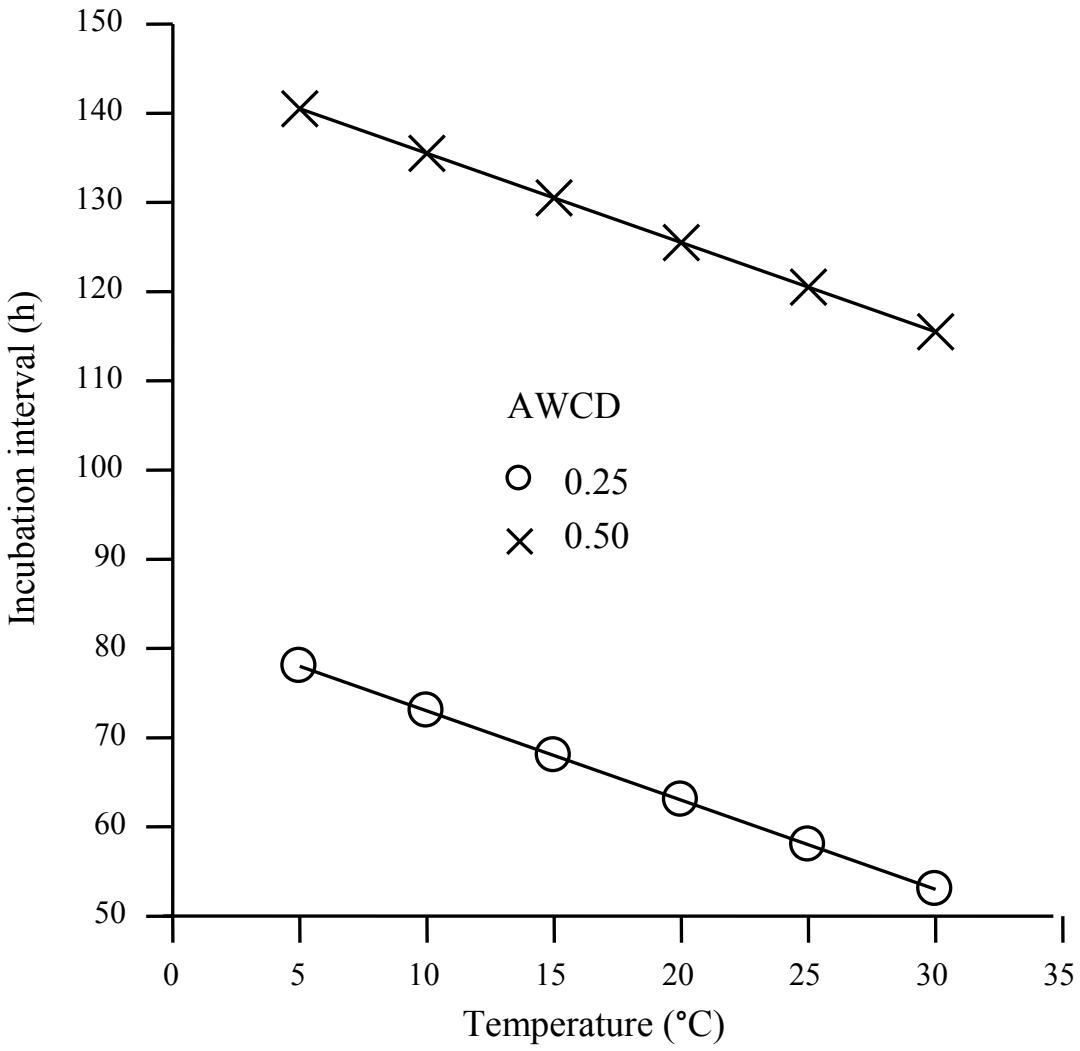

FIGURE 3.—Relationship between pond water sample temperature (°C) and incubation interval (h) required to achieve an average well color development (AWCD) value of 0.25 or 0.50. The linear regression equation of AWCD = -0.082 + 0.004*hours + 0.004* temperature was derived from 28 water samples from Possum Kingdom State Fish Hatchery ponds in November 2009 – May 2010 incubated on Biolog ™ EcoPlates.

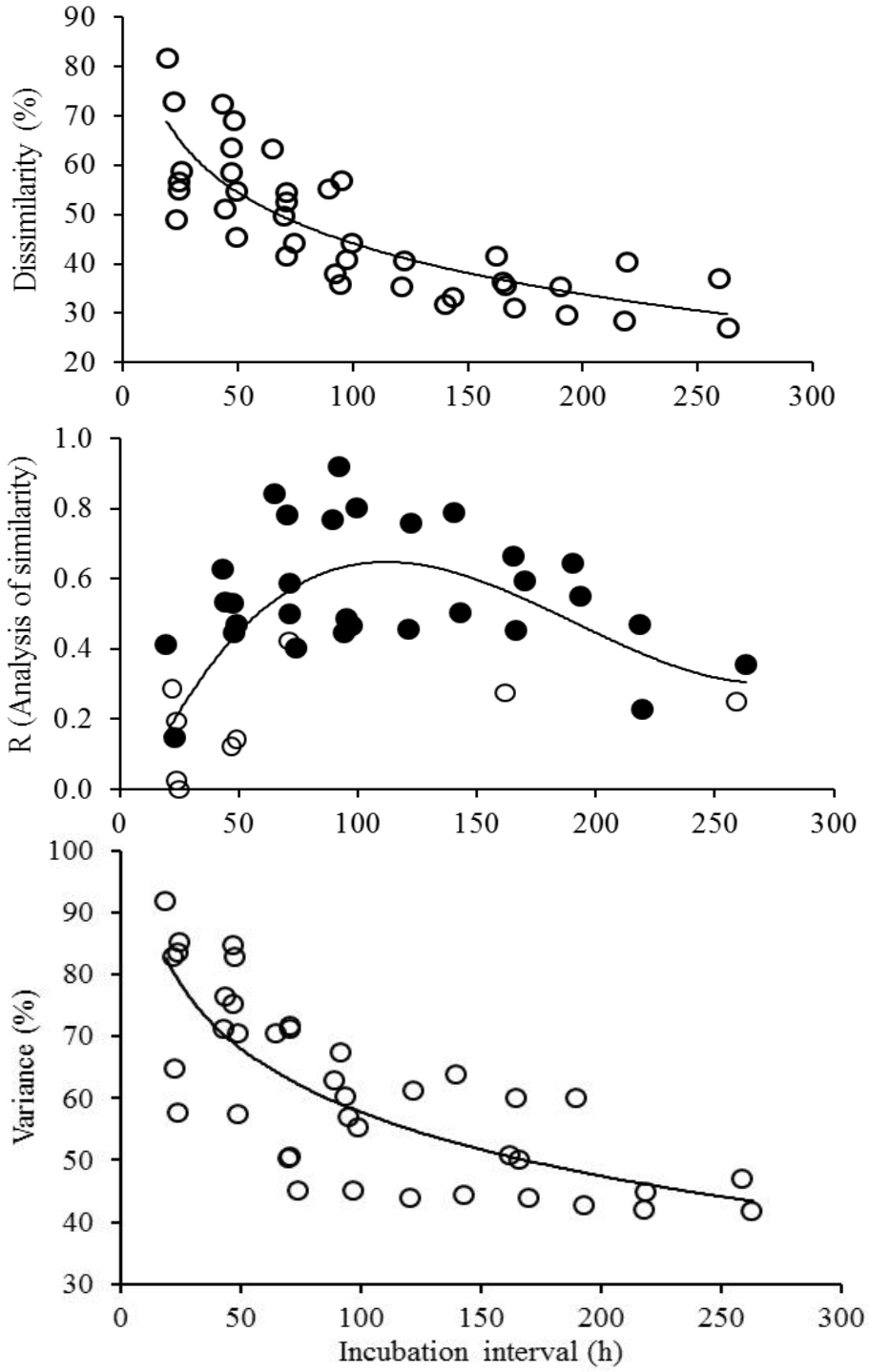

FIGURE 4.—Multivariate statistics of community substrate utilization patterns for 7 series of water samples from Possum Kingdom State Fish Hatchery ponds incubated on Biolog ™ EcoPlates in November 2009 – May 2010. Percent dissimilarity ranges from 0 (highly dissimilar) to 100% (highly similar) and *R* statistic ranges from 1 (completely separable communities) to 0 (inseparable communities). Open circles are not significant at $P \leq 0.05$. Variance is the percent of variance explained by the first two principle components.

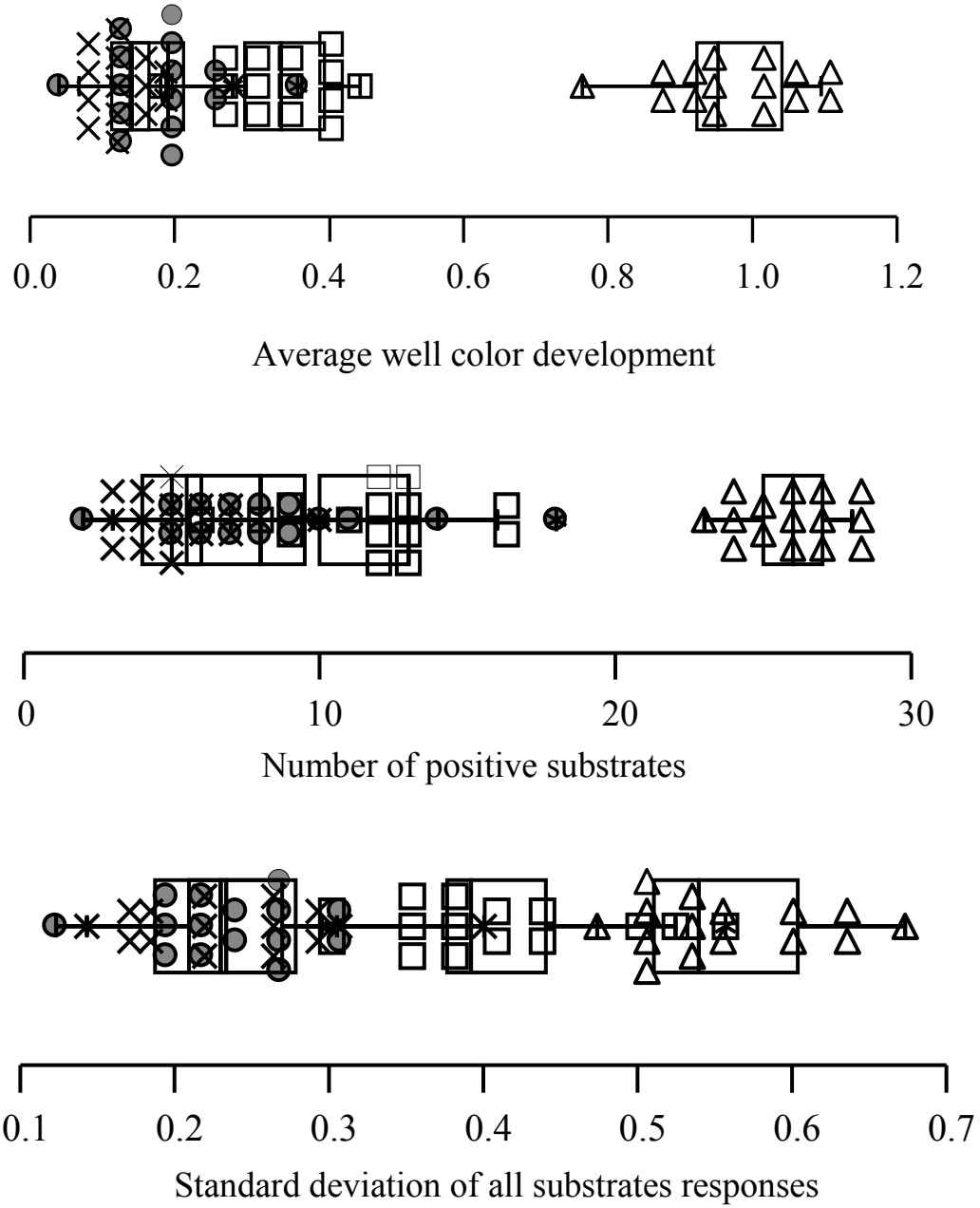

FIGURE 5.—Box plots of average well color development, positive substrate responses, and standard deviation of substrate responses for a single water sample collected from each of four Possum Kingdom State Fish Hatchery ponds (1, 2, 8, and 12) and incubated on 5 Biolog ™ EcoPlates to produce 15 replicates for each pond sample. Replicate symbols are: pond 1 - circles, pond 2 - Xs, pond 12 - squares, and pond 8 - triangles.

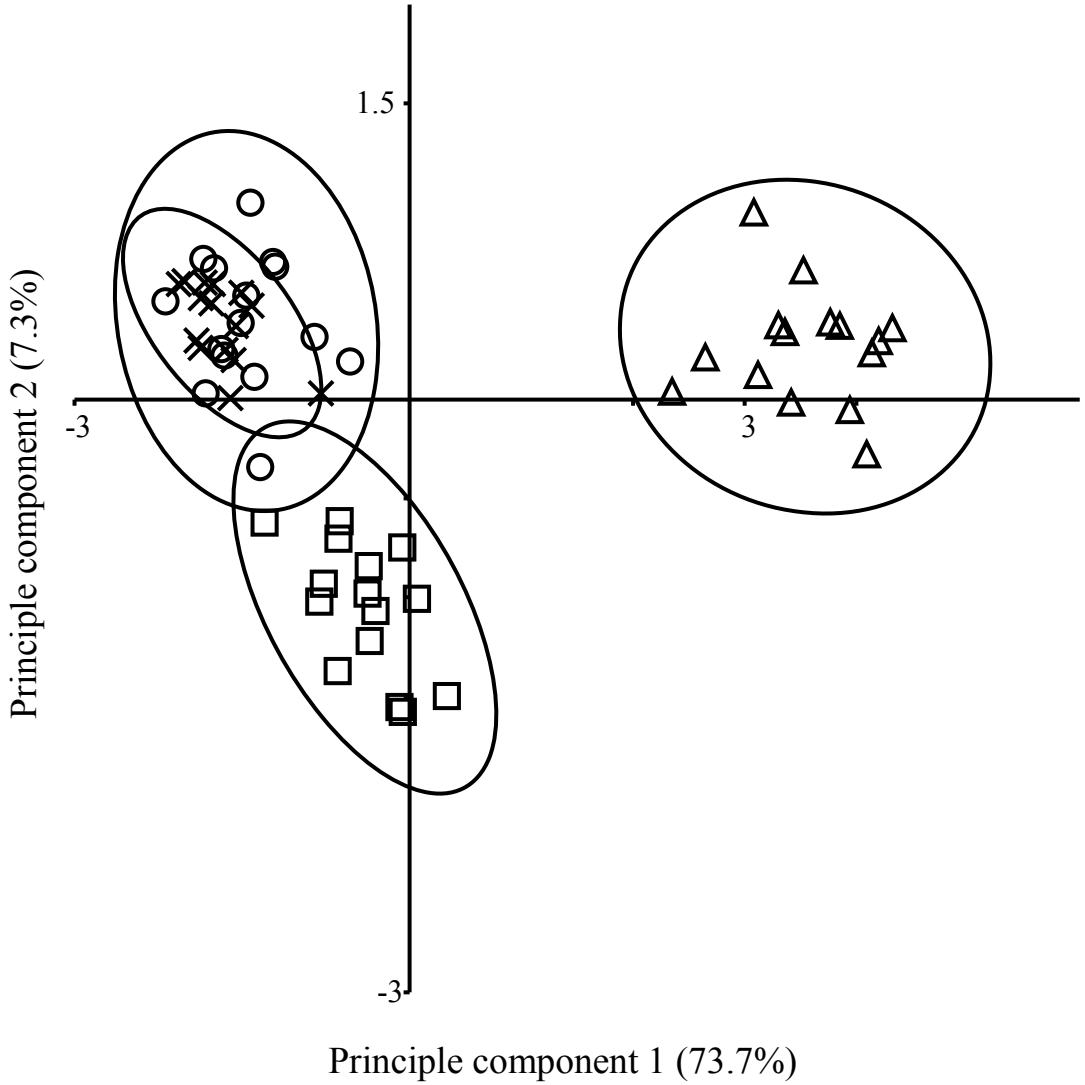

FIGURE 6.—Principle component ordination plot of 72-h community-level physiological profile responses of 15 replicates of water samples incubated on 5 Biolog ™ EcoPlates for each of 4 ponds sampled at the Possum Kingdom State Fish Hatchery. Ponds 1 and 2 were sampled on 26 May 2010 and 8 and 12 on 1 June 2010. Symbols are: pond 1- circles, pond 2 - Xs, pond 8 – triangles, and pond 12 - squares. Ellipses are the 95% confidence areas.

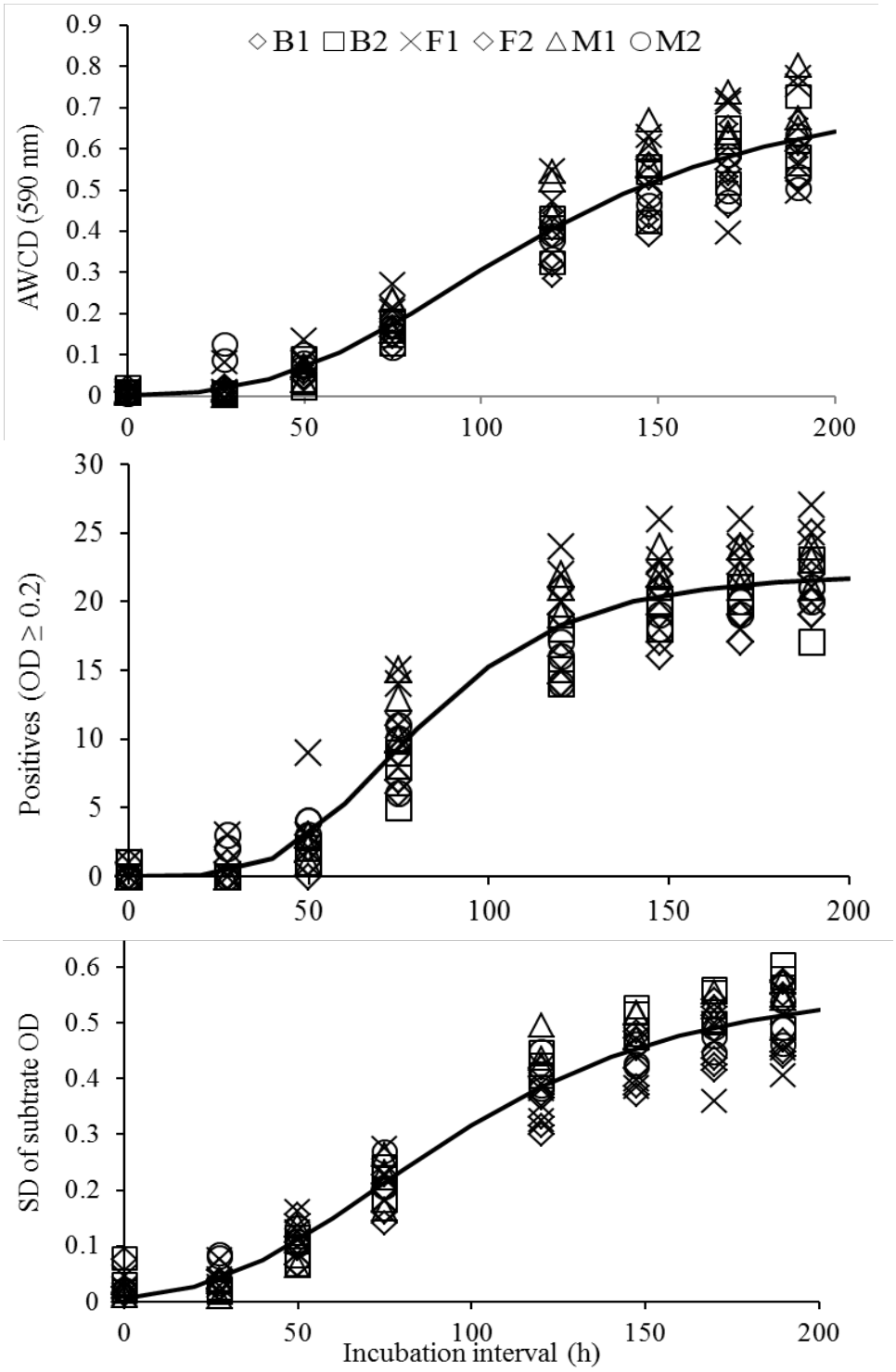

FIGURE 7.— Average well color development (AWCD), number of positive substrates, and standard deviation (SD) of substrate optical densities of community substrate utilization profiles for six water samples collected from pond 1 on 26 January 2010 at the Possum Kingdom State Fish Hatchery and incubated on Biolog ™ EcoPlates. Two water samples each (1 and 2) were collected from the front (F), middle (M), and back (B) of the pond. Each symbol is one of the three replicates of a single Biolog ™ EcoPlate.

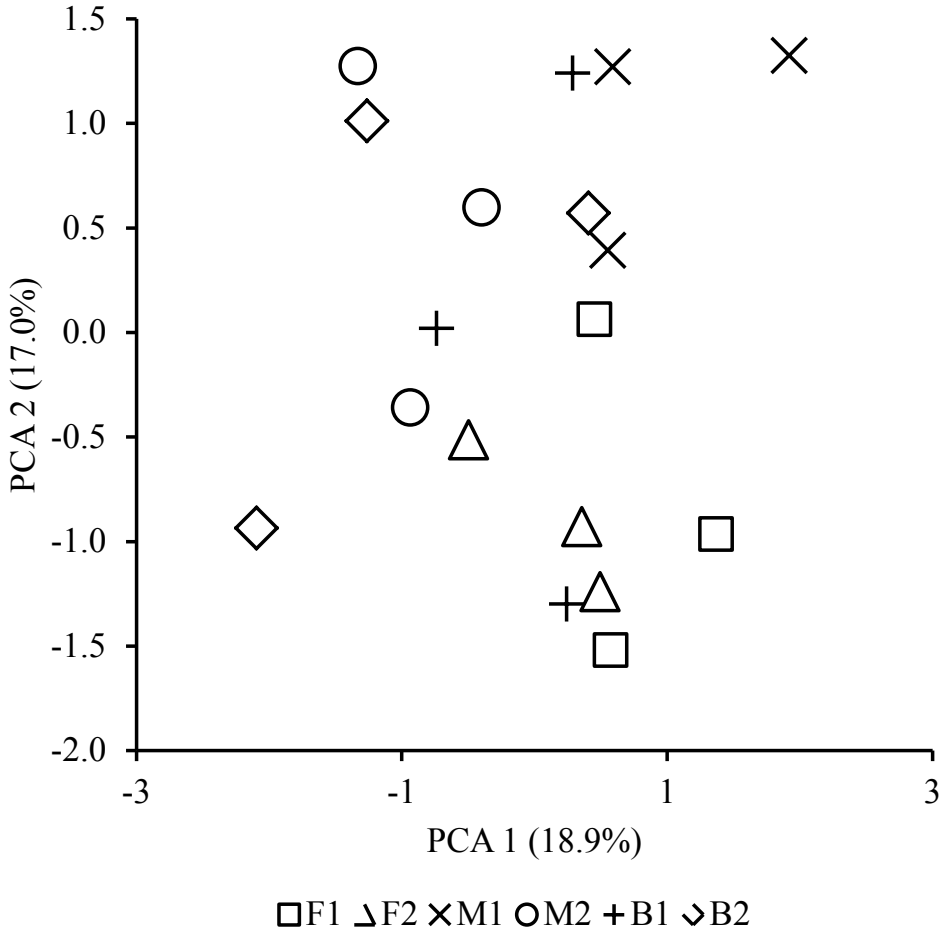

FIGURE 8.—Principle component plot of community substrate utilization profiles at 120-h incubation of 3 replicates each of 6 samples collected from pond 1 on 26 January 2010 at the Possum Kingdom State Fish Hatchery. Two water samples each (1 and 2) were collected from the front (F), middle (M), and back (B) of the pond.

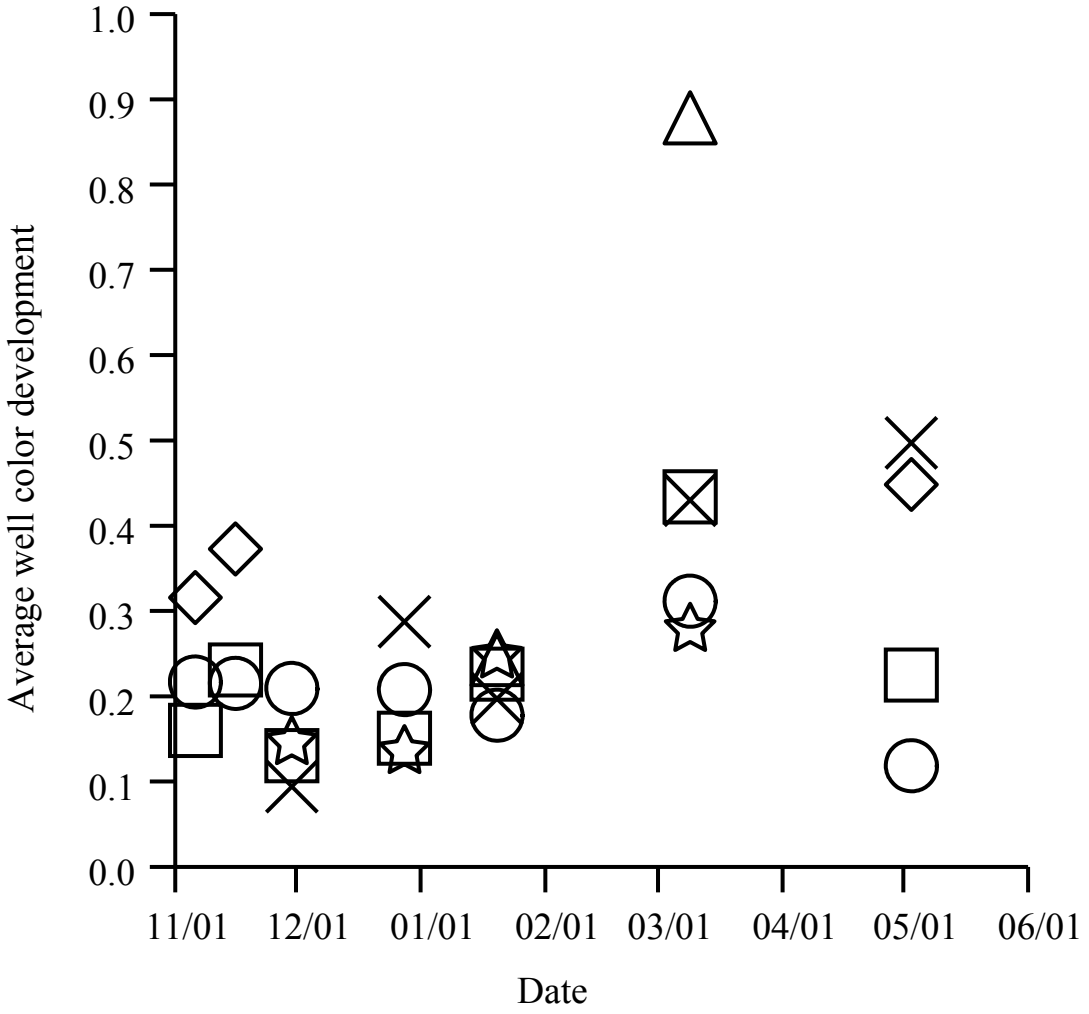

FIGURE 9.—Average well color development for six Possum Kingdom State Fish Hatchery ponds sampled up to seven times in November 2009 - May 2010. Each symbol is the mean of the three replicates for a single Biolog ™ EcoPlate. Symbols are: pond 1- circles, pond 13 - Xs, pond 15 - triangles, pond 19 - squares, pond 21 - diamonds, and pond 44 - stars.

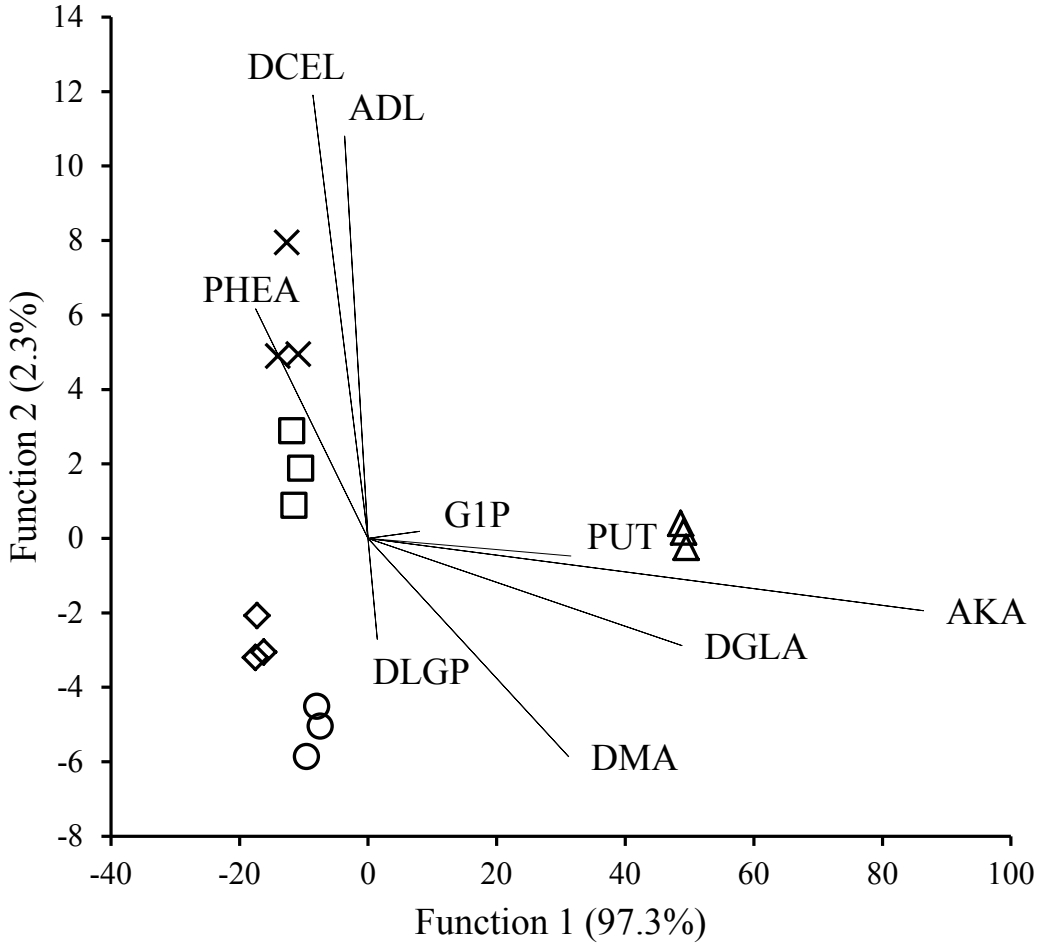

FIGURE 10.—Discriminant functional analysis plot of Biolog ™ EcoPlate substrate utilization profiles of water samples collected from five Possum Kingdom State Fish Hatchery ponds on 9 March 2010. Each symbol is one of the three replicates on each EcoPlate. Symbols are: pond 1- circles, pond 13 - Xs, pond 15 - triangles, pond 19 - squares, and pond 44 - diamonds. Function 1 is increased utilization of Glucose-1-phosphate (G1P), Putrescine (PUT), α-Ketobutyric acid (AKA), D-Glucosaminic acid (DGLA), and D-Malic acid (DMA). Function 2 is decreased use of D, L-α-Glycerol phosphate (DLGP) and increased use of Phenylethylamine (PHEA), D-Cellobiose (DCEL), and α-D-Lactose (ADL).

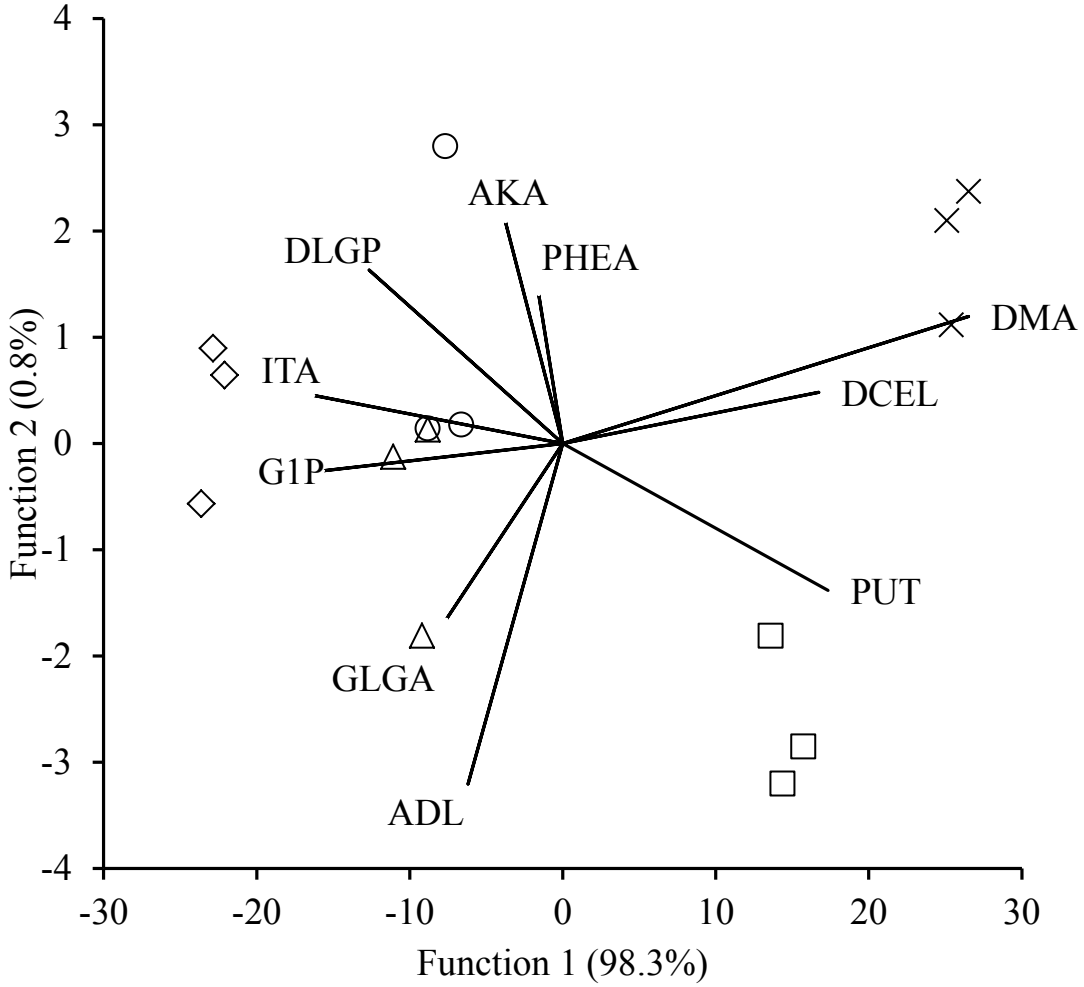

FIGURE 11.—Discriminant functional analysis plots of Biolog ™ EcoPlate substrate utilization profiles of water samples collected from five Possum Kingdom State Fish Hatchery ponds on 20 January 2010. Each symbol is one of the three replicates on each Biolog ™ EcoPlate. Symbols are: pond 1- circles, pond 13 - Xs, pond 15 - triangles, pond 19 - squares, and pond 44 - diamonds. Function 1 is increased use of D-Malic acid (DMA), D-Cellobiose (DCEL), Putrescine (PUT), and decreased use of Glucose-1-phosphate (G1P), Itaconic acid (ITA), and D, L-α-Glycerol phosphate (DLGP). Function 2 is increased use of α-Ketobutyric acid (AKA), Phenylethylamine (PHEA), and DLGP and decreased use of Glycyl-L-glutamic acid (GLGA) and α-D-Lactose (ADL).

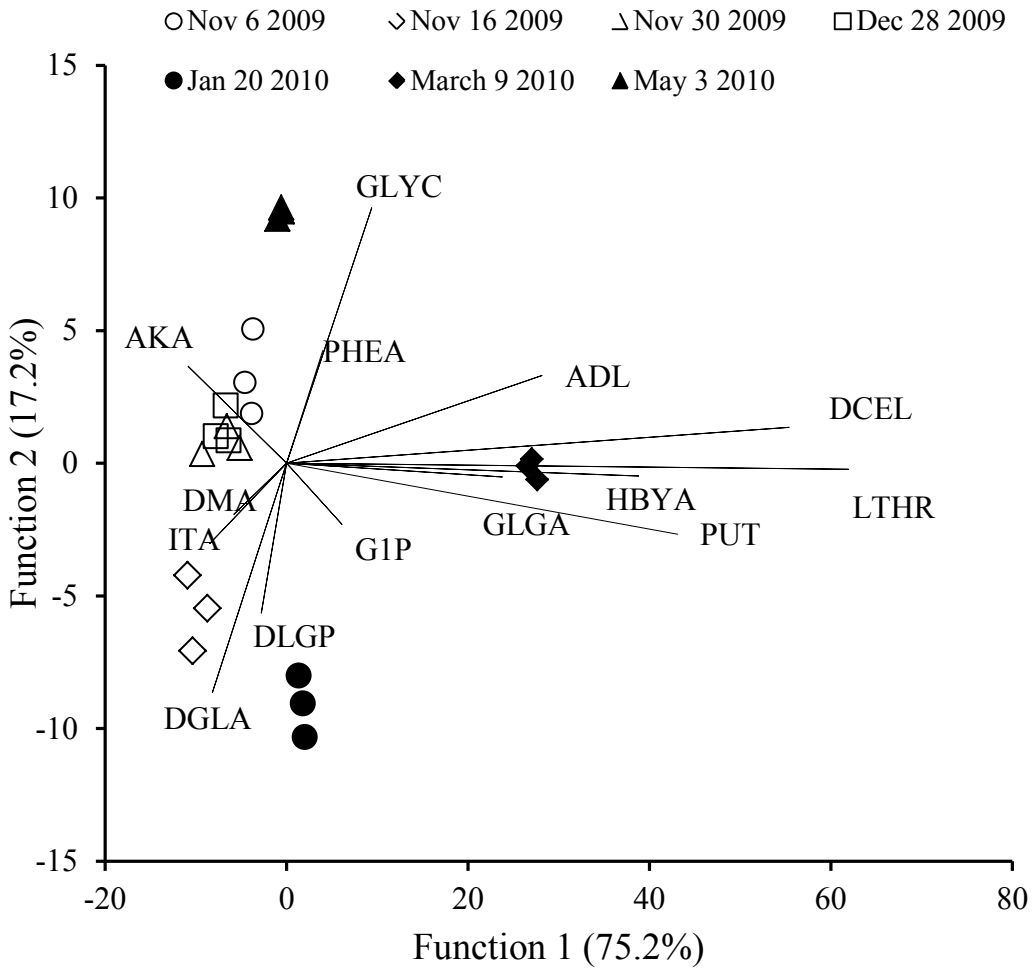

FIGURE 12.—Discriminant functional analysis plots of Biolog ™ EcoPlate substrate utilization profiles of water samples from Pond 19 at the Possum Kingdom State Fish Hatchery during seven seasonal sampling events. Each symbol is one of the three replicates on each EcoPlate™. Function 1 is increased use of D-Cellobiose (DCEL), L-Threonine (LTHR), Putrescine (PUT), γ-Hydroxybutyric acid (HBYA), α-D-Lactose (ADL), Glycyl-L-glutamic acid (GLGA), and Glucose-1-phosphate (G1P) and decreased use of α-Ketobutyric acid (AKA), D-Malic acid (DMA), and Itaconic acid (ITA). Function 2 is increased used of Glycogen (GLYC), Phenylethylamine (PHEA), and AKA and decreased use of D, L-α-Glycerol phosphate (DLGP), D-Glucosaminic acid (DGLA), G1P, DMA, and ITA.

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