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Title

Engineering difference: Matrix design determines community composition in wastewater treatment systems

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Abstract

There is a growing view that the application of ecological theory has the potential to facilitate a transition from a descriptive to a predictive framework in wastewater engineering. In this study we tested the hypotheses that: (i) it is possible to engineer consistent differences between microbial communities in wastewater treatment modules; (ii) there is a positive relationship between structural complexity and genetic diversity; (iii) such interactions are modulated by the availability of energy. We developed four treatment modules of increasingly complex support material (matrix) design, and pumped a synthetic wastewater through them for 16 weeks. We then disassembled the modules and assessed the phylogenetic (general eubacteria and ammonium oxidizers, by DGGE profiling) and phenotypic (by PLFA profiling) diversity of the communities present on the support materials. We found that different genotypic and phenotypic community structures were reliably generated by the engineering of their physical environment in terms of structural complexity (as determined by particle size distribution and therefore pore size distribution). Furthermore, there was a notably consistent response of the phenotypic structure to such circumstances, and also to the presence of organic matter. However, we found no significant relationships between genetic diversity and structural complexity either for eubacterial or ammonia-oxidiser microbial groups. This work demonstrates that is it possible to engineer modules of differing microbial community composition by varying their physical complexity. This is an essential first step in testing relationships between system diversity and treatment resilience at a process level.

Keywords

Wastewater treatment, engineered diversity, microbiological diversity, complexity, physical heterogeneity

1 Introduction

The ideal wastewater treatment process should use renewable resources in construction and little or no energy or other inputs in operation, and provide effective treatment to meet standards which protect the environment at the lowest possible economic and social cost (Griffin and Upton 1999). This aspiration has underpinned the widespread adoption of extensive or natural wastewater treatment technologies such as wetlands and ponds in recent years (Brissaud, 2007; Cooper, 2009). Such extensive treatment systems are subject to, and should be sufficiently robust to deal with, fluctuating environmental conditions such as freezing, variable hydraulic loading, and shock loads of treatable substances and toxins (Salvado *et al*, 2001; Werker *et al*, 2002; Molle *et al*, 2006). The design and maintenance protocols, for such treatment methods have been largely developed in an empirical manner, with no theoretical underpinning.

Tying sound engineering to good biological-ecological theory has recently been recognised as a way beyond a "trial and error" approach to system optimisation (Curtis *et al*, 2003; Todd *et al*, 2003; McMahon *et al*, 2007). The ability to engineer biologically based wastewater treatment systems to produce predictable and consistent community configurations has the potential to deliver more effective and efficient treatment systems, and is a long term goal of wastewater research (Curtis and Sloan, 2006). There is a growing view that the application of ecological theory has the potential to facilitate a transition from a descriptive to a predictive framework (Curtis *et al*, 2003; McMahon *et al*, 2007).

The design of resistant and resilient wastewater treatment systems might be possible using this approach and the putative relationship between biodiversity and system stability (Elton, 1958; McCann, 2000; Briones and Raskin, 2003; Hooper *et al*, 2005; Balvanera *et al*, 2006; McMahon *et al*, 2007; Wittebolle *et al*, *2009*) is of obvious relevance. Naeem and Li (1997) advanced the idea that biodiversity represents a form of biological insurance and that redundancy (multiple species per functional group) enables communities to maintain function during environmental change. Empirical evidence in support of the diversity-stability relationship has emerged from relatively recent work in terrestrial (Griffiths *et al*., 2001; Allison and Martiny 2008; Nielsen *et al* 2011) and aquatic ecosystems (Naeem and Li, 1997; von Canstein *et al*., 2002;) . Other factors, most notably abundance, may also affect the reliability of a functional group (Pickering, 2008).

Singer *et al*. (2010) in their work on dissolved organic carbon (DOC) use by streambed biofilms suggest a relationship between the fine-scale physical heterogeneity of a habitat and community diversity. In their work, more heterogeneous flow landscapes triggered greater bacterial biodiversity and biofilm architecture and ultimately led to the utilisation of a broader range of DOC compounds than in less physically-heterogeneous systems. It has also been demonstrated that one of the bases for the extreme levels of eukaryotic diversity found in soils relates to the structural heterogeneity of the pore networks and the resultant spatial isolation of microbes that ensues (Zhou *et al*. 2002). This finding suggests that it is possible to control the community diversity of a system by engineering its physical complexity. The

definition of structural complexity is a subject of great debate (e.g. Parrott, 2010). In this present study we define it as the range of niches available for colonisation. We postulate that the manipulation of system complexity/heterogeneity *in toto* (biological and physical diversity *sensu* Parrott, 2010) provides a potential mechanism by which wastewater treatment system stability may ultimately be engineered. A prerequisite for testing these concepts is the reliable production of discrete treatment units supporting differing microbial community compositions in a consistent manner. In this paper we set out to test the hypotheses: (i) that systems of differing microbial community composition can be generated by altering the physical complexity of a model wastewater treatment module matrix (support materials); and (ii) that there is a positive relationship between structural complexity and genetic diversity. To achieve this goal, we designed modules of differing physical complexity. We further hypothesised (iii) that such interactions are modulated by the availability of energy to the resultant microbial communities, and hence incorporated an organic-matter treatment (added straw) in the design.

2 Materials and methods

2.1 Experimental design

The experimental unit (module) was a 1.1-litre polypropylene cylindrical container packed with porous media as a base for biofilm development (Fig. 1). The modules were operated as submerged aerated filters treating a simple synthetic wastewater with ammoniacal-nitrogen as the principal treatment process indicator. The experiment involved four levels of matrix complexity, with an additional factor of organic matter amendment (i.e. with or without organic matter) imposed onto all complexity levels. The complexity levels were prescribed as minimal, low, medium or high , (representing an increase in the diversity of pore sizes within the modules), as denoted in Table 1. The organic matter amendment comprised 1g of wheat straw (1.5 mm milled). In addition 2 g of air-dried sandy soil (procured from Silsoe Farm, Bedfordshire, UK; 52°:00':29'' N: 0°:26':08''W; Cottenham Series) was added to all treatments as a common diverse microbial inoculum. The soil and straw were added to the base of each module. In the treatments based upon mixtures, aggregates were deposited into layers such that Caledonian gravel (roughly spherical, solid) was at the base, followed by Hydroleca (roughly spherical) then Glass Aggregate (spherical solid) and finally Glasscrete (spherical solid), where appropriate. Such sequences were repeated three times thereby giving three compounded layers. Three independent replicates of each module type were established. The synthetic wastewater solution consisted of 1.43 µM ammonium sulphate (i.e. 45 mg N l^{-1}) and 5.9 µM sodium bicarbonate dissolved in tap water and equilibrated at 17 ºC (±1 ºC). The solution was supplied to the base of each module at a rate of 0.5 ml min^{-1} using a peristaltic pump. Air was supplied to the base of each module at 30 litres min^{-1}. The experiment was incubated at 17ºC (±1 ºC) and maintained for 16 weeks.

Effluent was sampled weekly for ammonium-N, total oxides of nitrogen (TON), pH, alkalinity and conductivity using standard methods *(*APHA, 1998). Ammonium-N (automated phenate method;) and TON (automated hydrazine reduction method) were determined using the Burkard SFA 2000 auto-analyser. Alkalinity was determined by

titration using 0.02M HCl and Bromocresol green/Methyl red indicator. In-module dissolved oxygen was measured daily using a Hach HQ30d Meter, LDO101 Standard Dissolved Oxygen Probe (Hach, Loveland, CO).

After 16 weeks of operation all modules were consistently producing a nitrified effluent (ammonium-N <1 mg l^{-1}) and were consequently disassembled for sampling. The filter media were mixed, divided into thirds (including liquid) according to weight. Biomass was recovered from the residual liquid by filtration (sterile Whatman 0.2µm filter papers), and returned to the respective aliquots prior to analysis. The aliquots were analysed for microbial biomass, phenotypic and genotypic structure.

2.2 Analytical methods

2.2.1 Biomass carbon flush

Microbial carbon (C) was determined by modifying the BS 7755 fumigation-extraction technique (British Standards 1997). The matrix and associated filters were fumigated under chloroform for 24h at 20°C. Following fumigation, living biomass C was extracted by shaking for 1h with 0.5M potassium sulphate. Associated un-fumigated samples were also extracted to determine non-microbial carbon. Extracted C was determined using a Segmented Flow Analyser based upon UV digestion (Burkard Scientific SFA-2000 Segmented Flow Analyser: Burkhard, Herts, UK). We did not apply a correction factor to estimate microbial biomass since published factors relate to soil matrices.

2.2.2 DNA analysis

DNA was extracted from the matrices using the FastDNA SPIN Kit for soil (Qbiogene, Cambridge, UK), according to the manufacturer's instructions.

2.2.3 Polymerase chain reaction

The target gene fragments were amplified using the polymerase chain reaction (PCR), using 16-S based ribosomal RNA eubacterial- or ammonia-oxidising bacterial (AOB) specific primers. For eubacteria, the primers comprised (from 5'end to 3' end): CCTACGGGAGGCAGCAG; ATTACCGCGGCTGCTGG; Muyzer *et al*., 1993), and for AOB primer sequences were GGAGRAAAGYAGGGGATCG; CTAGCYTTGTAGTTTCAAACGC; Kowalchuk *et al*., 1997). Forward primers contained the GC- clamp – CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG. Each sample for PCR amplification contained 1 µl of template DNA; 1 µl of forward and reverse primer (10 p-moles per µl); and 47 µl of MegaMix-Blue (Microzone, West Sussex, UK). For eubacteria PCR conditions were 3 min, 95oC initial denaturation followed by 30 s, 95°C; 1 min, 65°Cb; 1 min, 72°C (24 cycles); 30 s, 95°C; 1 min, 53°C; 1 min, 72°C (15 cycles); and final extension 10 min, 72°C. For AOB, PCR conditions were 3 min, 95°C initial denaturation followed by 30 s, 95°C; 1 min, 57°C; 1 min, 72°C (30 cycles); final extension 10 min, 72°C. PCR amplification reactions were performed using a Perkin Elmer DNA Thermal Cycler (Bucks, England).

2.2.4 Denaturing gradient gel electrophoresis (DGGE)

This technique was conducted as described by Muyzer *et al*. (1993), with the exception that either a 10% (w/v) or a 7% (w/v) polyacrylamide gel were used with a range of denaturants at 30-60% (w/v). PCR-amplified samples were directly applied onto a 10% (w/v) or a 7% (w/v) polyacrylamide gel in 0.5×TE (20 mM Tris acetate at pH 7.4, 10 mM sodium acetate, 0.5 mM Na2-EDTA). The gradient in the gel was formed with 10% (w/v) or a 7% (w/v) acrylamide stock solution (acrylamide-N,N'-methylenebisacrylamide, 37:1), which contained 30% and 60% denaturant (7 M urea) and 40% (v/v) formamide deionised with AG501-X8 mixed-bed resin, using a D-Gene system (Biorad, Hemel Hempstead, UK) . Gels were run for 4 h at 200V constant voltage and at 60°C and subsequently stained for 30 minutes in SYBR green I (Sigma, Poole, UK). The presence, relative location and intensity of the resultant bands were analysed using Bionumerics 4.0 (Applied Maths BVBA, Saint-Martens-Latem, Belgium).

2.2.5 Phenotypic community structure

Phospholipid fatty acid (PLFA) analysis was used to assess the phenotypic structure of the microbial communities using the method of Frostegård *et al*. (1993). Phospholipids were extracted from aliquots representing one-third of the resultant matrices using chloroform, methanol and citrate buffer to the ratio of 1:2:0.8 (v/v/v), fractionated by solid phase extraction, depolymerised and then derivatised by mild alkaline methanolysis. The resultant fatty acid methyl esters were analysed by gas chromatography (Agilent Technologies, Santa Clara, California, USA).

2.3 Statistical analyses

The dimensionality of the multivariate DGGE and PLFA profiles was reduced using unconstrained principal component (PC) analysis using the correlation matrix. The Shannon-Wiener Index was calculated for DGGE profiles (Magurran, 1988). PCs, diversity indices and C-flush data were analysed by two-way analysis of variance (ANOVA). *Post-hoc* Fisher multiple range test was applied to determine heterogeneous groups of means, using Statistica v 9.1.

3 Results and discussion.

3.1 Biomass carbon flush

Carbon flush is a measure of the total amount of biomass in the system. Structural complexity and straw did influence C-flush following chloroform fumigation but no significant interaction (Table 2) was observed. There was a greater C-flush from Minimal-complexity modules than in the other three types, regardless of the presence of straw (Fig. 2). This is counter-intuitive as it might be expected greater opportunity existed for biomass development in relation to surface area, which increased with module complexity. The addition of straw resulted in a greater C-flush (1.54 versus 2.61 µg C g^{-1} matrix for no-straw and plus-straw treatments respectively, pooled SE 0.18), demonstrating that the additional C added in the form of straw resulted in a greater microbial biomass, as would be expected.

3.2 DGGE profiles

There were no significant interactions between structural complexity and straw addition upon eubacterial profiles for any of the first three PCs, which collectively accounted for 40% of the variance (Table 2). PC1-3 all discriminated significantly between the different forms of structural complexity (Table 2) with PC1 separating Low- from High- complexity modules, and PC2 Minimal-complexity modules from the others (Fig. 3A). PC3 also significantly separated Low-complexity modules from the other three, which were otherwise similar (data not shown). Therefore the differences in the eubacterial community would appear to be controlled by complexity of the modules.

The Shannon diversity index for eubacterial profiles was significantly greater in the presence of straw (2.27 versus 2.51 for no-straw and plus-straw treatments respectively; pooled SE 0.063, $P<0.05$). Whilst statistically significant, such a difference is comparatively small in the context of this index. This suggests that there were more species when additional substrate was provided, which we would expect. There was no effect of structural complexity upon the Shannon index (Table 2). This then does not support the hypothesis that greater structural diversity necessarily results in greater phylogenetic diversity, but does confirm that community phenotypic composition is sensitive to such properties, at least in terms of the eubacterial community.

In relation to ammonia-oxidiser community profiles, there was a significant interaction between structural complexity and straw addition for PC1 and a direct straw effect for PC2 (Table 2). In the absence of straw, there were essentially two classes of genetic profile, *viz.* those associated with Minimal- and Low-complexity, and those with Medium- and High-complexity modules (Fig. 3B). Therefore the complexity of the modules has produced distinctly different AOB communities in the absence of straw amendment. However, when straw was present, profiles associated with the Medium-complexity modules were distinct from the other three module types (Fig. 3B). Given that the effect was manifest only in the Medium-complexity modules, its basis is unclear.

The Shannon diversity index for AOB profiles was smaller in High-complexity modules than the other three types, which were otherwise similar (1.01 for High versus 1.33, 1.35, 1.44 for Medium, Low and Minimal modules respectively, pooled SE 0.09). Again, this does not apparently support the hypothesis of a positive relationship between genetic diversity *per se* and structural complexity. There was no significant effect of straw addition upon the AOB diversity index (Table 2), which would be expected, as these organisms are primarily autotrophs and not therefore reliant upon an energy source from the added organic matter.

3.3 Phenotypic profiles

There were no significant interactions between structural complexity and straw addition upon the phenotypic profiles for any of the first three PCs, which collectively accounted for 60% of the variance. There was a highly significant effect of the presence of straw upon the PLFA profiles, which dominated PC1 (Table 2). There was however, clear separation of module types with respect to phenotype in PCs 2 and 3 (Fig. 4). There was a strikingly consistent

trajectory in PC2 with respect to structural complexity, with the Minimal- and High-complexity modules being at the two extremes and the other modules intermediate (Fig 4A). The High-complexity modules were then distinct in relation to PC3 (Fig 4B). The addition of organic matter therefore had a dominant effect upon the phenotypic structure in all cases, which indicates that the microbial phenotype was strongly influenced by the presence of additional substrate. There was then a consistent effect of structural complexity upon the phenotype, revealed by the apparent trajectory in PC2 and distinctness of the High-complexity treatment in PC3. The microbial phenotypic structure was therefore consistently responsive to both the presence of organic matter in the microcosm and its structural context. We suggest this is because the phenotype is essentially a manifestation of the genotype:environment interaction, and therefore has a high fidelity to the environmental context in which it develops.

3.4 General discussion

The effect of structural complexity upon the bacterial phylogenetic diversity is not apparently in accord with previous data in relation to structure:complexity relationships, despite there being significant effects upon other properties. One of the mechanisms by which such diversity arises is the spatial isolation of microbes in small-scale physical niches (Zhou *et al* 2002), which can be attenuated by connection via water films (Zhou *et al* 2002; Long and Or 2009). Since the modules in this study were saturated, such isolation mechanisms are unlikely to have operated. Furthermore, in microbial communities a large proportion of the community is commonly to be found in resting/quiescent structures (Llorrens *et al*, 2010). This is not analogous to the composition of communities of plants and animals in most ecosystem studies. Although plants do have substantial dormant phases (seeds), these are rarely measured in studies of function-diversity relationships – usually such work focuses on adults, and over relatively short time scales (e.g. Cardinale, *et al*, 2006). Therefore, we suggest that direct comparisons may only be drawn between microbial community phenotypes and those ecological studies where complexity:diversity relationships have been derived. We cannot of course account for those taxa that fall below the detection threshold of the methods employed. However, since those taxa will account for less than 1% of the biomass (Muzer *et al*., 1993) they will not have a significant effect on the ammonia or carbon removal in an engineered system.

4 Conclusions

This study strongly supports our first hypothesis that differing microbial community compositions, both in relation to the phylotype and phenotype can reliably be generated by engineering their physical environment in terms of structural complexity. Furthermore, there was apparently a notably consistent response of the phenotypic structure to such circumstances. The second hypothesis of a positive relationship between phylogenetic diversity and structural complexity was not supported either for general (eubacterial) or specific (AOB) microbial groups. This work demonstrates that we can engineer modules of differing microbial community composition by varying their physical complexity. This is an

essential first step in testing relationships between system diversity and treatment resilience at a process level.

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Table 1: Module composition.

Complexity level	Total mass (kg) of matrix added			
	Caledonian Pebbles[1]	Glass Aggregate[2]	Glasscrete[3]	Hydroleca[4]
Minimal	2			
Low	1	0.75		
Medium	0.66	0.5	0.56	
High	0.56	0.357	0.425	0.125

[1]Scottish granite river-stone pebbles (approx. diameter 20mm), Product code 2404; [2]Recycled glass (approx. diameter 6-12mm), Trade name Enviro-Glass-AQUA, Product code 7408; [3]Sand manufactured from recycled glass (approx diameter 1-2mm), Product code 3014; [4]Lightweight expanded clay aggregate (LECA) granules produced from fired clay (approx. diameter 8-12mm), Product code 7892. All aggregates were procured from Specialist Aggregates Ltd, Stafford, UK.

Table 2. Significance levels for ANOVA of data relating to C-flush following chloroform fumigation, principal component (PC) analysis of denaturing gradient gel electrophoresis (DGGE) profiles for eubacteria and ammonia oxidising bacteria with associated Shannon indices, and phospholipid fatty acid (PLFA) profiles.

Property		ANOVA term			Error mean-square
		Complexity	Straw	Complexity x straw	
C-flush		**	***	ns	0.37
DGGE – Eubacteria	PC1	*	ns	ns	4.0
	PC2	*	***	ns	2.4
	PC3	*	ns	ns	3.0
Shannon index		ns	*	ns	0.047
DGGE – Ammonia oxidising bacteria	PC1	ns	ns	*	3.2
	PC2	ns	*	ns	1.5
	PC3	ns	ns	ns	0.44
Shannon index		*	ns	ns	0.049
PLFA	PC1	ns	***	ns	4.1
	PC2	**	ns	ns	3.4
	PC3	**	ns	ns	1.4

ns = $P > 0.05$; * = $P < 0..05$; ** = $P < 0.01$; *** $P < 0.001$

Figure captions

Figure 1: Diagrammatic representation of the experimental design

Figure 2. C-flush following chloroform fumigation of solid phases of modules in relation to structural complexity of such phases. Bars show means across modules with and without straw (n=6); whiskers denote pooled standard errors; letters denote similar means using Fisher's multiple range test.

Figure 3. Principal component (PC) plots based upon genotypic (DGGE) profiles of microbial communities in relation to structural complexity of modules. (A) Eubacteria. Min = minimal, Lo = Low, Med = Medium, Hi = high complexity respectively. Points show means across modules with and without straw incorporated (n=6); whiskers denote pooled standard errors. (B) Ammonia-oxidising bacteria. Bars show means (n=3); whiskers denote pooled standard errors; letters denote similar means using Fisher's multiple range test. Percent variance accounted for by PCs shown in parentheses on axis labels.

Figure 4. Principal components (PCs) based upon phenotypic (PLFA) profiles of microbial communities in relation to structural complexity of modules. (A) PC2. (B) PC3. Bars show means across modules with and without straw (n=6); whiskers denote pooled standard errors; letters denote similar means using Fisher's multiple range test. Percent variance accounted for by PCs shown in parentheses on axis labels respectively.

Figure 1

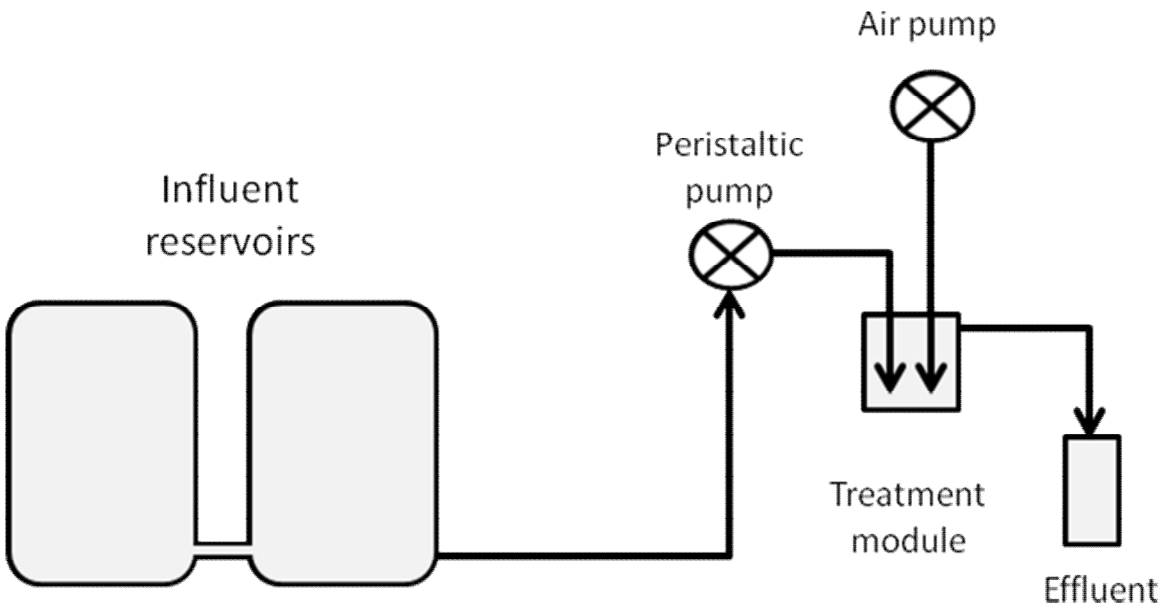

Air pump
Peristaltic pump
Influent reservoirs
Treatment module
Effluent

Figure 2

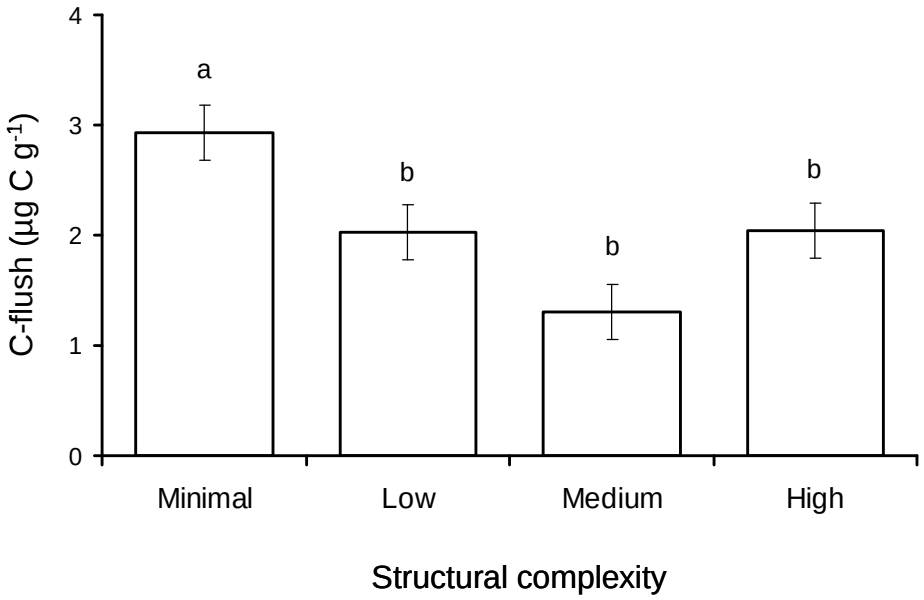

4
3
2
1
0
C-flush (µg C g-1)
a
b
b
b
Minimal
Low
Medium
High
Structural complexity

Figure 3

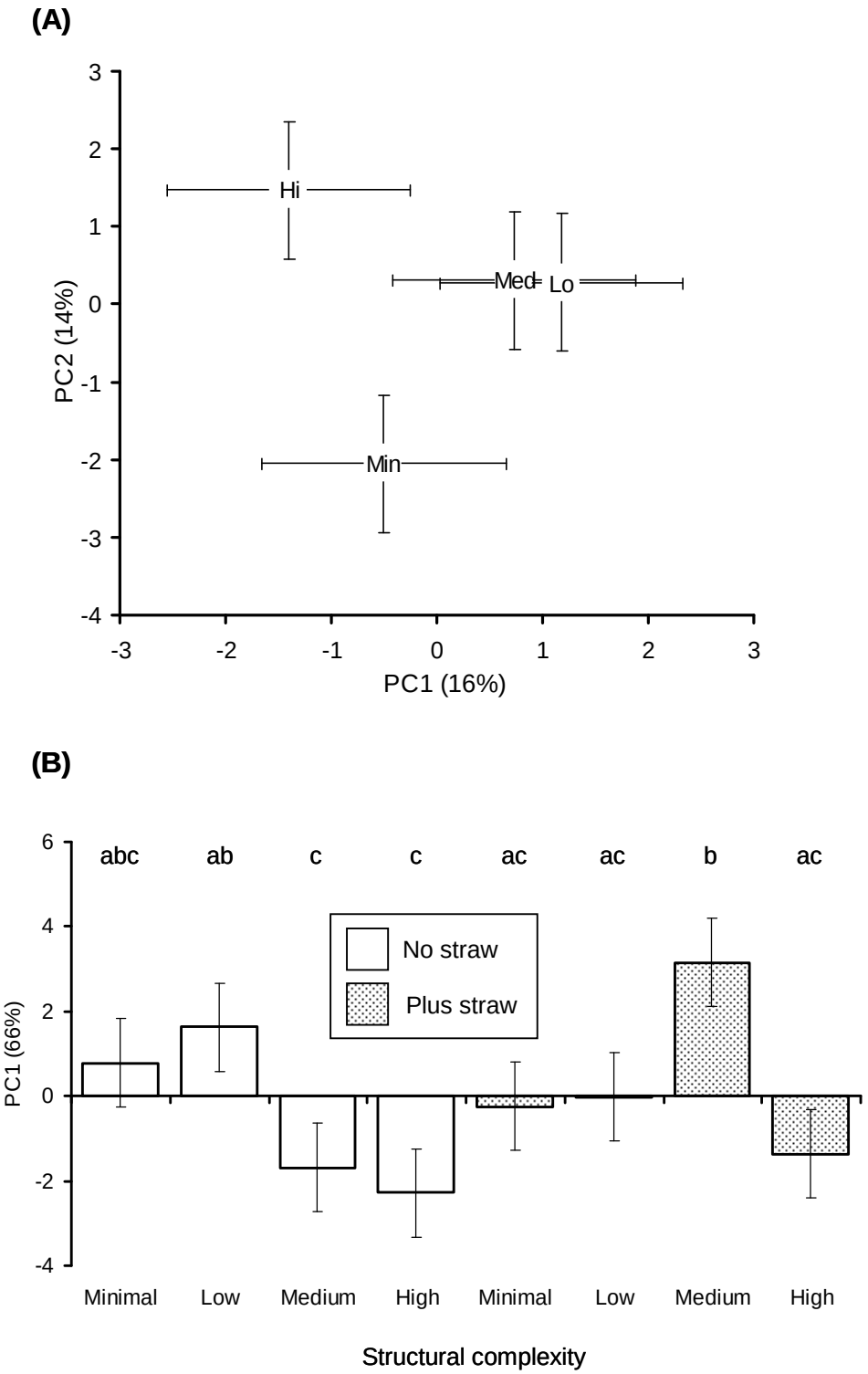
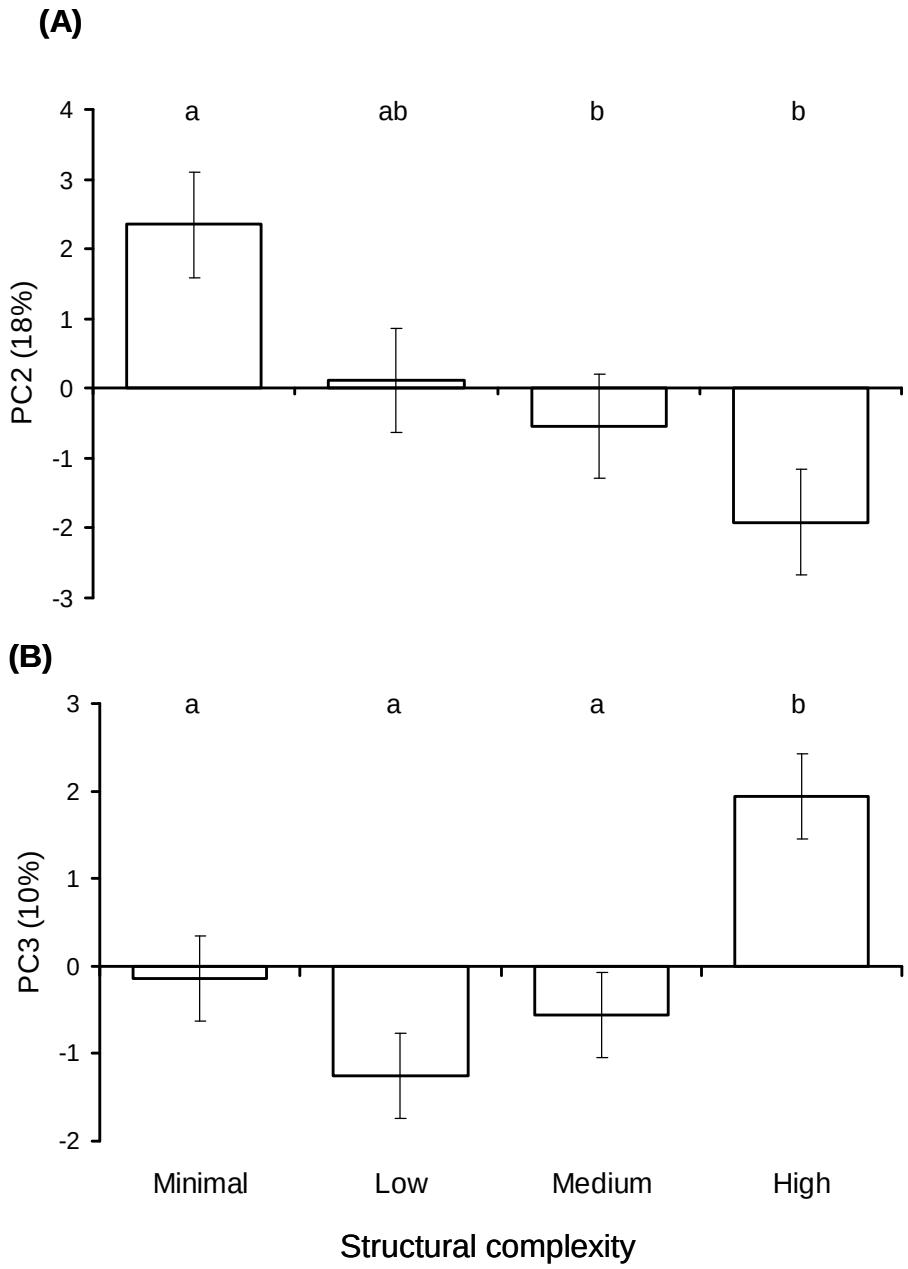

(A)
Hi
Med
Lo
Min
PC2 (14%)
PC1 (16%)
(B)
abc
ab
c
c
ac
ac
b
ac
No straw
Plus straw
PC1 (66%)
Minimal
Low
Medium
High
Minimal
Low
Medium
High
Structural complexity

Figure 4


(A)
a
ab
b
b
4
3
2
1
0
-1
-2
-3
PC2 (18%)
(B)
a
a
a
b
3
2
1
0
-1
-2
PC3 (10%)
Minimal
Low
Medium
High
Structural complexity