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Nathalie Pautremat

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THESE

Université d'Avignon et des Pays du Vaucluse
ECOLE DOCTORALE ED380 SCIENCES ET AGRONOMIE
DOCTORAT HYDROGEOLOGIE (mention: Biogéochimie)

Conséquences du recyclage agricole
de déchets agro-industriels liquides et fermentescibles
sur la mobilité des métaux préexistants dans le sol
(Fe, Mn, Cr, Ni)

Interactions entre microbiologie et réactivité géochimique abiotique

Nathalie Pautremat

Soutenue le 24 octobre 2005

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I Introduction générale

650 Mt de déchets ont été produits en France en 2001, dont 50 % ont été recyclés par la filière agricole (IFEN, 2002). En 2000, 13 M m³ d'effluents liquides, dont 4 M m³ d'effluents des secteurs du sucre et de distillerie, avaient été épandus sur des sols agricoles (MEDD, 2002). L'apport d'effluents liquides riches en matières organiques favorise l'installation de conditions anaérobies transitoires. L'anaérobiose permet de réduire les pollutions par NO₃⁻ (Parry et al., 2000), par des composés organochlorés (Liu et al., 1991) et des hydrocarbures (Zocca et al., 2004), et peut aboutir à l'immobilisation de certains radionucléides et métaux lourds (Lloyd, 2003). Cependant, elle est susceptible d'accroître les émissions de N₂O et CH₄ qui sont des gaz à effet de serre (Conrad, 1996), la production et l'accumulation de composés toxiques (NO₂⁻ (Roy et Conrad, 1999) et acides organiques (Zehnder et Stumm, 1988)) et la mobilisation de certains éléments métalliques (Quantin et al., 2001). La mobilisation de métaux peut avoir des conséquences écotoxicologiques néfastes sur les organismes du sol (Rajapaksha et al., 2004) et sur les plantes (Hue et al., 1999). Si cette mobilisation s'accompagne du transfert de ces éléments, elle peut éventuellement affecter la qualité de l'aquifère sous-jacent (Charlatka et al., 1995).

En condition d'anaérobiose, les activités microbiennes sont à l'origine de la plupart des évolutions géochimiques (Zehnder et Stumm, 1988). Résultant de microorganismes présentant une grande diversité fonctionnelle, elles affectent (i) le niveau d'oxydoréduction en jouant sur différents couples redox en solution (NO₃⁻/NO₂⁻, H⁺/H₂, NO₂⁻/NH₄⁺, Fe³⁺/Fe²⁺, SO₄²⁻/HS⁻, CO₂/CH₄...) (Peter et Conrad, 2000) et en réduisant directement certains éléments métalliques contenus dans les phases solides (Dong et al., 2003), (ii) les conditions acido-basiques, par la production ou la consommation directes de H⁺ (par exemple au cours de la réduction de NO₂⁻ en N₂O) de CO₂, de NH₄⁺, de HS⁻, d'acides organiques (Stumm et Morgan, 1996), et (iii) la complexation des métaux (Rue et Bruland, 1995; Jansen et al., 2002) *via* l'accumulation de certains composés minéraux (HCO₃⁻, HS⁻, NH₃...) et organiques (formate, acétate, propionate, butyrate etc.) pouvant complexer les métaux (Glissman et Conrad, 2000). Ces évolutions géochimiques induisent des réactions abiotiques qui affectent en retour les activités microbiennes. Les réactions abiotiques tamponnent les variations de pH (Kimura,

2000), incluent la complexation d'éléments métalliques, et certaines réactions redox ; elles mettent en jeu de nombreuses réactions entre solution et phases solides (dissolutions/précipitations (Lefèvre-Drouet et Rousseau, 1995; Scheidegger et Sparks, 1996), adsorptions/désorptions (Marschner et Kalbitz, 2003)).

Cependant l'anoxie est souvent temporaire et/ou d'extension spatiale limitée (Parry et al, 2000, Brune et al., 2000). La juxtaposition temporelle ou spatiale entre anaérobiose et aérobie aboutit à des fonctionnements spécifiques du sol, dus à la présence simultanée de composés réduits et oxydés (rouilles vertes, NO_3^- , NO_2^- , NH_4^+ ...) (Hansen et al., 1996 ; Murase et Kimura, 1997). Ces variations d'états d'aération du sol posent des questions relatives au maintien des pollutions initiées par l'anaérobiose, et aux résiliences microbienne et chimique après un retour à l'aérobiose. A notre connaissance, peu d'études traitent de la résilience géochimique (van Bodegom, 2001) minéralogique (Duckworth et Martin, 2004; Edwards et al., 2000) ou microbienne (Fareleira et al., 2003) pour ce type de fluctuations.

L'objectif de notre travail était de contribuer à l'évaluation des risques de mobilisation d'éléments métalliques préexistants dans les sols (Fe, Mn ...) suite à un apport de déchets liquides chargés en composés fermentescibles. Au vu du temps imparti à ce travail, nous nous sommes focalisés sur l'étude de la réactivité biogéochimique, en abordant successivement 3 thèmes :

- L'extension de travaux décrivant les interactions entre microbiologie anaérobie et géochimie abiotique pour un sol amendé en glucose et NO_3^- (Dassonville et al., 2004a, 2004b) à des apports au sol de mélanges complexes (sucres, protéines, lipides, acides organiques, acides aminés, phénols ...). Notre objectif était plus précisément de hiérarchiser les nouveaux besoins de description des grandes voies du catabolisme anaérobie en relation avec la réduction et la mobilisation des métaux ;
- La réduction dans le sol en anaérobiose de FeIII et de MnIV avec (i) les contributions relatives des réductions biologiques directes et abiotiques à ces réductions, (ii) pour les réductions biologiques directes l'impact de différents types de donneurs d'électrons (H_2 , acides organiques, sucres) et accepteurs d'électrons (NO_3^- , SO_4^{2-}) et (iii) la physiologie des réducteurs de FeIII (aptitude à croître sans réduction de FeIII et choix éventuel entre réduction de FeIII et autres catabolismes pour ceux aptes à dénitrifier, réduire le SO_4^{2-} et/ou fermenter), les études passées relatives à ces réductions, très nombreuses, étant soit à dominante géochimique, soit à dominante microbiologique et,

dans ce cas, abordant souvent le problème à partir de souches isolées (Ona-Nguéma, 2003) ;

- l'étude du maintien en solution de FeII et de MnII mobilisés au cours d'un épisode anaérobie, suite à un retour à des conditions aérobies, ainsi que des possibilités de résiliences chimique et microbienne du sol, les alternances 'anaérobiose – aérobiose' n'ayant fait l'objet, à notre connaissance, que de très peu de travaux pour les sols, la littérature étant plus abondante pour les sédiments de surface sujet à des variations temporelles de profondeur de la zone aérée (Brune et al., 2000).

Nos travaux étaient basés sur des incubations anaérobies en batch d'un Ferralsol de La Réunion riche en Fe, Mn, Cr et Ni, dû à son origine volcanique, amendé avec de la vinasse de rhumerie. Les analyses visaient à suivre les biotransformations et les dynamiques microbiennes, ainsi que certaines évolutions géochimiques en solution, voire en phases solides.

Ce mémoire présente l'ensemble des résultats obtenus lors de cette étude. Il est structuré en 5 parties :

- Un 1^{er} chapitre synthétise les acquis scientifiques préalables à notre travail et disponibles dans la bibliographie ; elle traite des volets biologiques et abiotiques de la réactivité du sol en relation avec la réduction et l'oxydation de Fe et Mn, en accordant plus de place au volet microbien ;
- Un 2nd chapitre traite des interactions entre microbiologie anaérobie et géochimie abiotique dans le cas d'apport de vinasses de rhumerie ;
- Un 3^{ème} chapitre est dédié à la réduction de FeIII et MnIV en anaérobiose dans le cas d'apport de vinasses de rhumerie ;
- Un 4^{ème} partie présente les résultats d'expériences sur le retour à l'aérobiose après une première incubation en anaérobiose ;
- Enfin, les acquis et des questions nées de ce travail de thèse sont réunis, résumés et discutés dans un 5^{ème} chapitre où nous tentons par ailleurs de faire quelques propositions de recherche ultérieure.

Tableau 1 : Teneurs totales en éléments traces dans les sols

| Eléments Métalliques | MONDE | | FRANCE (Baize, 2000) | | LA REUNION (Doelsch, 2004) | |
|-------------------------|--|------------------------------------|---|---|---|------------------------------|
| | mg kg ⁻¹ terre fine (< 2 mm) | Gamme de valeurs habituelles | Gammes de valeurs Anomalies naturelles modérées | Gammes de valeurs Anomalies naturelles accentuées | 75 % des échantillons | Ensemble des échantillons |
| | | | mg kg ⁻¹ terre fine (< 2 mm) | | mg kg ⁻¹ terre fine (< 2 mm) | |
| As | 1-20 | 1.0-25.0 | 30-60 | 60-284 | | |
| Cd | 0.2-1.0 | 0.05-0.45 | 0.7-2.0 | 2.0-16.0 | 0.0-0.3 | 0.0-0.8 |
| Cr | 70-100 | 10-90 | 90-150 | 150-3180 | 35.0-480.5 | 35-1108 |
| Co | 10 | 2-23 | 23-90 | 105-148 | | |
| Cu | 20-30 | 2-20 | 20-62 | 65-102 | 6.5-82.3 | 6.5-164.4 |
| Hg | 0.03-0.06 | 0.02-0.10 | | | 0.0-0.3 | 0.0-0.8 |
| Mn | | | | | 594.9-2676.7 | 594.9-8066.9 |
| Ni | 50 | 2-60 | 60-130 | 130-2076 | 15.1-305.3 | 15-1037 |
| Pb | 10-30 | 9-50 | 60-90 | 100-3000 | | |
| Se | 0.5 | 0.10-0.70 | 0.8-2.0 | 2.0-4.5 | | |
| Tl | - | 0.10-1.7 | 2.5-4.4 | 7.0-55.0 | | |
| Zn | 50 | 10-100 | 100-250 | 250-3800 | 57.5-205.9 | 57.5-398.3 |

II Synthèse bibliographique

II.1 Déchets liquides fermentescibles et agriculture : contextes environnemental et agronomique

La loi française du 13 juillet 1992, avec l'interdiction progressive du stockage des déchets non ultimes, a abouti à la systématisation des pratiques de valorisation et à l'intégration des déchets comme produits à part entière de l'industrie, de l'agriculture et des communautés urbaines. Plus récemment, la réglementation européenne du 1^{er} juillet 2002 en matière de protection de l'environnement a stipulé l'obligation de ne mettre en décharge que des déchets ultimes (déchets qui ne sont plus susceptibles d'être traités dans les conditions techniques et économiques du moment). L'épandage est ainsi communément utilisé pour la valorisation des déchets liquides provenant de l'industrie alimentaire, tels que la production d'huile d'olive (Zenjary et Nejmeddine, 2001), les distilleries de vin ou de rhum (Gemtos et al., 1999), les brasseries (EC, 2001), et les laiteries (Shi et al., 1999). L'épandage de ces effluents combine un apport riche en matières organiques et en éléments nutritifs (EC, 2001). Toutefois, selon la circulaire du 17 décembre 1998, l'épandage de ces déchets ne peut être pratiqué que sur des sols de pente inférieure à 7 % (ce chiffre peut quelque peu différer selon la nature du sol et la végétation).

Du fait de la nature liquide et organique de ces déchets, les risques d'anaérobiose sont transitoirement accrus (Renault et Sierra, 1994) en raison d'une consommation accrue d'O₂ dont la diffusion est réduite en parallèle. Si l'anaérobiose a des effets positifs sur l'élimination de NO₃⁻ (Parry et al., 1999), des pesticides chlorés (Liu et al., 1991) et des composés phénoliques (Lovley, 1991), elle est aussi susceptible de provoquer (i) un accroissement des émissions de gaz à effet de serre - N₂O et CH₄ - (Conrad, 1996), (ii) une production et/ou une accumulation de composés toxiques dans le sol dont NO₂⁻ (Roy et Conrad, 1999) et certains acides organiques (Zehnder et Stumm, 1988), et (iii) la mobilisation d'éléments métalliques (Quantin et al., 2001). De telles conditions peuvent alors conduire au transfert de ces derniers éléments vers les eaux souterraines. Les éléments Fe, Mn et d'autres ETM peuvent être naturellement abondants dans certains sols (Tableau 1) ou apportés avec les épandages (Speir

Tableau 2 : Effets du pH et du potentiel redox sur la mobilité des ETM (Förstner, 1985).

| Mobilité relative | pH | | E_H | |
|-------------------|------------------------|------------------------|------------------------|-----------------------------------|
| | Neutre -Alcalin | Acide | Oxydant | Réducteur |
| Très haute | Se, Mo | B | B | |
| Haute | As | Zn, Cd, Hg, Co, Ni, Mn | Mo, Se | |
| Moyenne | Mn | Cu, Al, Pb, Cr | Cu, Co, Hg, Ni, Zn, Cd | Mn |
| Basse | Pb, Fe, Zn, Cd, Tl | FeIII, Tl | Pb, Tl | FeII, Zn, Co, Ni, Tl |
| Très basse | Al, Cr, Hg, Cu, Ni, Co | Mo, Se, As | Al, Cr, Fe, Mn | Al, Cr, Hg, Cu, Se, B, Mo, Cd, Pb |

Tableau 3 – Analyses physico-chimiques de vinasses de rhumerie.

| | Vinasses – Distillerie Isautier La Réunion (Feder et al., 2004) | Analyses de vinasse (littérature*) Moyenne (Ecartype) |
|--------------------------|---|---|
| DCO (g / l) | 103 | |
| pH | 4,6 | 4,30 (0.54) |
| conductivité (mS / cm) | 38,4 | |
| densité à 20°C | 1,054 | |
| % de MS | 11,3 | 8,10 (2.45) |
| MO tot. (g pour 100g MS) | 66 | |
| N tot. (g pour 100g MS) | 2,81 | 1,81 (0.83) |
| P (g pour 100g MS) | 0,23 | 0,29 (0.27) |
| K (g pour 100g MS) | 13,8 | 10,65 (3.21) |
| Ca (g pour 100g MS) | 2,18 | 1,78 (0.26) |
| Mg (g pour 100g MS) | 1,17 | 1,05 (0.15) |
| Cl (g pour 100g MS) | | 5,38 (1.55) |
| Fe (mg pour 100g MS) | 716 | |
| Mn (mg pour 100g MS) | 171 | |
| Zn (mg pour 100g MS) | 40,8 | |
| Cu (mg pour 100g MS) | 25,3 | |

*Littérature :Pichot 1983 ; Mazza et al., 1986 ; Bontemps, 1987 ; Bazile et Bories, 1989 ; Matibiri, 1996 ; SUAD, 1996 ; MVAD, 1998.

et al., 2003). La mobilité des éléments métalliques varie avec le métal (Tableau 2) et les conditions du milieu (pH, potentiel redox, nature et teneurs des ligands minéraux et organiques) (Förstner, 1983). Dans les sols, les processus anaérobies sont cependant temporaires et limités dans l'espace (Sierra et Renault, 1998 ; Parry et al., 2000 ; Bidet et al., 2000). Lors d'un retour à l'aérobiose, le maintien ou non des éléments métalliques en solution pondèrera l'ampleur des risques associés à l'anaérobiose. Ainsi, au vu de la lente ré-oxydation abiotique de MnII, la mobilisation de cet élément apparaît plus préoccupante que celle d'autres éléments comme FeII, dont l'oxydation par voie abiotique est très rapide à pH 7 (Morgan, 2005 ; Stumm et Morgan, 1996).

Certains éléments métalliques, tels que Fe et Mn sont essentiels aux processus biologiques des microorganismes et des plantes. Pour parer à la faible solubilité des oxydes du sol, les plantes (Hisinger, 1998) et les microorganismes (Ams et al., 2002) peuvent sécréter des sidérophores permettant de mobiliser et complexer les métaux, afin de les assimiler ultérieurement. Toutefois, une mobilisation excessive des éléments métalliques (suite à un épisode anaérobie par exemple pour Fe et Mn) engendre des risques de pollution des sols, des eaux de surface et souterraines. On peut alors parler de risques écotoxicologiques pour l'homme (Doran et al., 2000), la macro- et la micro-faune du sol (Rajapaksha et al., 2004), les plantes (Ponnamperuma, 1972; Seaker, 1991), et les microorganismes (Adams et Ghiorse, 1995). Pour l'homme, les textes législatifs de janvier 1989 fixent les seuils critiques de Fe et Mn dans l'eau à 0.2 mg.L⁻¹ et 50 µg.L⁻¹. A titre d'exemples pour les microorganismes, un arrêt de la croissance en *Saccharomyces cerevisiae* est observée pour des teneurs en manganèse supérieures à 0.5 mM de MnII dans la solution du sol (Blackwell et al., 1998), tandis que pour une concentration supérieure à 55 µM dans l'environnement, MnII inhibe la croissance et le développement des cellules de *L. discophora* SS-1 (Adams et Ghiorse, 1995).

La production de canne à sucre est la principale activité de l'île de La Réunion. La canne est transformée par deux sucreries et les mélasses résultant de leur transformation sont distillées pour la production du rhum. En 2000, la Réunion produisait ainsi 119 000 hL d'alcool pur, engendrant la production de 150 000 t an⁻¹ de vinasse. La valorisation agronomique de l'eau et des nutriments contenus dans cet effluent (Tableau 3) apparaît d'autant plus appropriée que les pratiques actuelles de rejet en mer ou d'injection dans le sous-sol pourraient avoir des effets néfastes (Martial et al., 2002). A La Réunion, seulement 22 % (557 km²) de la superficie de l'île correspondent à des sols de pente inférieure à 10 %

(Raunet, 1991). Compte tenu que l'urbanisation, les terres réellement utilisables pour l'épandage représentent une faible superficie de l'île. Cette contrainte est amplifiée par la concurrence d'autres déchets, tels que le lisier de porc (240 000 t an⁻¹), les lisiers de bovins (200 000 t an⁻¹), les boues de station d'épuration, les déchets verts, etc. Si l'application de la loi du 13 juillet 1992 n'est pas encore effective, les villes, les industries et les agriculteurs sont d'ores et déjà soumis à « une pression réglementaire » qui induit une forte demande en matière d'expertise, de recherche et de transfert de technologie. Parmi ces différents risques environnementaux suite à la pratique de l'épandage (mobilisation de métaux, lixiviation de NO₃⁻, polyphénols..., effets sur les propriétés physico-chimique du sol) et vis-à-vis des différents types de déchets susceptible d'être épandus, nous nous focaliserons sur les risques de mobilisation des métaux suite à un apport de vinasses de rhumerie. De plus, ce problème fait intervenir différents volets, tels que la réactivité des minéraux, les activités microbiennes et plus particulièrement la respiration directe des métaux par les réducteurs de FeIII, ainsi que des conséquences d'aération. Nous ne tiendrons pas compte des transferts pour privilégier la réactivité.

II.2 Réactivités biologiques et abiotiques des éléments métalliques soumis à des alternances entre conditions réductrices et oxydantes

II.2.1 Forme et réactivité de Fe et Mn dans les sols

Les sols sont constitués de deux types de minéraux; les minéraux primaires (quartz, feldspaths, micas etc..) et les minéraux secondaires (argiles, oxydes), qui sont le résultat de transformations biogéochimiques de certains minéraux primaires (Duchaufour, 1997). Les phases solides porteuses de Fe ou de Mn incluent des oxydes (Schwertmann, 1985), des hydroxydes, des silicates, des carbonates et des composés soufrés (plus polysulfures que sulfates pour des éléments comme Fe ou Mn) (Stumm et Morgan, 1996; Pankow, 1991). Ils sont couramment sous forme de grains distincts d'hématite, magnétite ou goethite dans une matrice de quartz. Les principaux oxydes et oxy-hydroxydes du fer (Cornell et Schwertmann, 2003) rencontrés dans les sols sont l'hématite α -Fe₂O₃; la goethite α -FeOOH ; la maghémite γ -Fe₂O₃ ; la magnétite Fe₃O₄ ; la lépidocrocite γ -FeOOH ; la ferroxhydryte δ '-FeOOH et la

ferrihydrate $5\text{FeOOH}\cdot 2\text{H}_2\text{O}$. En aérobiose, les oxydes et silicates métalliques, peu altérables, sont libérés de la roche par érosion physique et/ou chimique et transportés sous forme de particules. Les oxydes présentent une grande variabilité de cristallinité, entre la goethite et l'hématite qui forment des structures bien cristallisées tandis que la ferrihydrate présente des défauts (Schwertmann, 1985). Cependant la réactivité des oxydes est d'autant plus élevée que les minéraux tendent vers une faible cristallinité (Larsen et Postma, 2001). Par ailleurs, les métaux présents dans les roches sous forme de sulfures et de carbonates peuvent être plus facilement altérés chimiquement et dissous dans l'eau.

Les charges négatives dominent généralement le complexe d'échange des sols. On distingue les charges permanentes, indépendantes du pH, dues aux substitutions isomorphiques dans les minéraux argileux et les charges variables, dépendantes du pH, dues en partie à la fixation de protons et en partie aux hydroxyles des oxydes métalliques et en bordure des feuillets argileux. Les métaux, ETMs et Fe, Mn, s'adsorbent sur les oxydes ou le complexe argilo-humique par échange ionique (liaison électrostatique) ou par liaison covalente. De nombreux facteurs peuvent influencer le phénomène d'adsorption des métaux sur les phases solides, tels que le pH, le nombre de sites de liaison, l'encombrement stérique des phases solides. Les métaux peuvent également être (i) incorporés dans la structure des réseaux silicatés, (ii) par substitution du FeIII dans les oxydes par d'autres cations de rayon ionique proche (tels que Al, Cr, Ni, Co), ou (iii) précipité ou coprecipité sous formes minérales (Banfield et Hamers, 1997 ; Webster et al., 1998; Martinez and McBride, 1998), dont les principales formes sont les oxy-hydroxydes de Fe et de Mn, les carbonates et les minéraux argileux.

En condition aérobie, Fe et Mn (à l'état FeIII et MnIV) sont relativement peu solubles dans l'eau (Cornell et Schwertmann, 2003) et restent majoritairement associés à la phase solide. Au contraire, l'anaérobiose favorise leur mobilisation par réduction microbienne (Straub et al., 2001). L'ampleur et la cinétique de la réduction microbienne des métaux dépendent de la disponibilité des accepteurs d'électrons, de la cristallinité des minéraux (Phillips et al., 1993), de leur granulométrie ou surface spécifique (Roden et Zachara, 1996), ainsi que de l'accessibilité aux éléments métalliques. Le fer contenu dans les argiles peut également être réduit par voie microbienne (Boivin et al., 2002 ; Favre, 2002, Favre, 2004) ; la réduction du fer structural des argiles engendre des modifications importantes des propriétés chimiques et physiques des argiles (Stucki, 1997), dont notamment l'augmentation

de leur charge de surface des argiles et de la capacité d'échange cationique (C.E.C.) (Stucki et Roth, 1977 ; Favre, 2002).

La Réunion est une île volcanique intraplaque formée de deux massifs : le Piton des Neiges qui est un volcan inactif et le Piton de la Fournaise, qui est un volcan récent toujours en activité (Raunet, 1991). Les sols réunionnais cultivés sont principalement acides ($4.5 < \text{pH} < 6.5$) (Doelsch, 2004) et présentent de fortes teneurs en ETM, particulièrement pour les éléments Cr, Cu, Ni et Zn (Tableau 1). Les concentrations en ETM et en éléments majeurs dans les sols dépendent fortement de l'origine et de la composition des roches mères, qui évolue avec l'activité des volcans de l'île. Nos expériences ont portées sur du sol prélevé de la station expérimentale du Cirad, nommée La Mare. Cette station expérimentale se situe dans la commune de S^{te} Marie dans le nord de l'île. Il s'agit d'un sol ferralitique rouge fortement à moyennement désaturé, issu des matériaux volcaniques non effondrés du Piton des Neiges de phase III de coulées (Raunet, 1991).

II.2.2 Devenir de Fe et Mn avec la mise en place de conditions réductrices

II.2.2.1 Activités microbiennes anaérobies et évolutions géochimiques

En condition anaérobie, les activités microbiennes sont le moteur initial des évolutions géochimiques (Zenhder et Stumm, 1988 ; Dassonville et Renault, 2002). En l'absence d'O₂, différents processus cataboliques alternatifs sont susceptibles d'être réalisés par les microorganismes :

- des respirations avec d'autres accepteurs terminaux d'électrons : quelques composés organiques (fumarate ...) (Loneragan et al., 1996), les oxydes de N (NO₃⁻, ...) (Tiedje, 1988), les formes oxydées de certains éléments métalliques (FeIII, MnIV ...) (Ghiorse, 1988), les oxydes de S (SO₄²⁻, ...) (Widdel, 1988), CO₂... (Oremland, 1988), ainsi que de nombreux autres composés généralement présents à l'état de traces (à titre d'exemples (Lovley, 1991)) ;
- des processus fermentaires (Colberg, 1988 ; McInerney, 1988) et acétogènes (Dolfing, 1988).

Les activités microbiennes affectent ainsi :

- i/ le potentiel d'oxydoréduction du milieu en jouant sur différents couples redox ($\text{NO}_3^-/\text{NO}_2^-$, $\text{NO}_2^-/\text{NH}_4^+$, FeIII/FeII , $\text{SO}_4^{2-}/\text{H}_2\text{S}$, CO_2/CH_4 ...) (Peter et Conrad, 2000) ;
- ii/ le pH de la solution du sol au travers de la production ou la consommation directe de protons (Tiedje, 1988 ; Legall et Fauque 1988), de la production nette de CO_2 et d'acides organiques (Kirama, 1989 ; Stumm et Morgan, 1996), ainsi que de la dissolution ou précipitation de minéraux, notamment d'oxyhydroxydes (Dommergues et Mangenot, 1970) ;
- iii/ la production de ligands minéraux (NH_3 , OH^- , HCO_3^- , CO_3^{2-} , HS^- , S^{2-}) (Ponnamperuma, 1972) et organiques (acides organiques, acides aminés ...) complexant les métaux (Millero et al., 1995; Strathmann et Myeni, 2004; Ge et al., 2000).

Les microorganismes participent indirectement à la mobilisation des métaux *via* leur action sur l'installation de conditions réductrices, le pH de la solution et les productions nettes de ligands minéraux et organiques. Certaines réductions abiotiques peuvent se faire à des vitesses similaires aux réductions biologiques. Ainsi et à titre d'exemples la réduction de métaux couplées à l'oxydation des sulfures en S^0 , de S^0 en SO_4^{2-} (Murase et Kimura, 1997), ou de NO_2^- en NO_3^- (Komatsu et al., 1978) ne peut être négligées. La réduction abiotique des éléments métalliques dépend du pH et varie notablement selon la nature de la matière organique et de sa capacité à acidifier le milieu et à complexer les métaux. Généralement la réduction biologique prévaut sur la réduction abiotique (Nealson et Little, 1997).

II.2.2.2 La réduction microbienne anaérobie des métaux

Jusqu'à récemment, la réduction des éléments métalliques était considérée comme le résultat de processus abiotiques (Ghiorse, 1988). Cependant dès la fin des années 70, on a isolé plusieurs souches de micro-organismes anaérobies aptes à croître en satisfaisant leur besoin énergétique par la réduction d'éléments métalliques (FeIII , MnIV ...) (Münch et Ottow, 1983). Depuis, il est devenu évident que la mobilisation des métaux peut résulter de la réduction microbienne directe des éléments métalliques contenus dans les oxy-hydroxydes (Lovley et al., 1993) et les argiles (Favre et al., 2002). La réduction du FeIII et du MnIV s'effectue soit par contact direct entre les oxydes du sol et les bactéries (Das et Caccavo, 2000), soit par « des navettes à électrons » (telles que les substances humiques (Kappler et al.,

2003), AQDS (anthraquinone-2,6-disulfonate) (Ona Nguema, 2003)), qui agissent comme transporteurs d'électrons entre les micro-organismes et les oxydes (Lloyd, 2003), soit par complexation ou chelation des métaux favorisant leur mobilisation (Kendall et Hochella, 2003) comme les sidérophores (Hernandez et Newman, 2001).

La nature des oxydes métalliques affecte les cinétiques de réduction. En effet, les oxydes amorphes sont plus rapidement réduits que les formes cristallines (Phillips et al., 1993 ; Lovley, 1995 ; Glasauer et al., 2003). Par exemple, des études menées en rizières (Liesack et al., 2000) ont révélé une réduction préférentielle de la ferrihydrite et de la lépidocrocite (minéraux de faible cristallinité et considérés comme peu métastables), bien que ces sols contiennent également de la goethite et de l'hématite. Des expériences comparant la réduction microbienne par *Shewanella alga* BrY de FeIII amorphe, de goethite et d'hématite ont montré que 20 fois plus d'oxyde de FeIII amorphe que de goethite et 50 fois plus de goethite que d'hématite ont été réduits lors d'une incubation de 5 jours (Roden et Zachara, 1996). De plus la nature des oxydes métalliques affecte également la croissance des FeIII-réducteurs. Les cinétiques de croissance de *Shewanella putrefaciens* sur smectite et lépidocrocite étaient 2 à 3 fois plus importantes que la vitesse de croissance sur goethite et magnétite (Kostka et al., 2002). Les cinétiques de réduction du MnIV dépendent également de la nature des oxydes disponibles (Burdige et al., 1992). De même, la granulométrie des phases solides, ou plutôt la surface spécifique des oxydes associés, affecte également la capacité des microorganismes à réduire les métaux. Roden et Zachara (1996) ont montré que les cinétiques de réduction microbiennes des oxydes étaient contrôlées par la surface spécifique et la concentration en site de réaction des phases solides. Par conséquent, l'adsorption de matières organiques, et d'anions minéraux (phosphates, SO_4^{2-} ...) sur les phases solides peut au contraire limiter les contacts entre les microorganismes et les phases solides, ou le transfert d'électrons. Les ions phosphates ont une forte tendance à s'adsorber sur les oxydes de fer et à complexer les espèces ioniques du fer (Borggaard et al., 2004). L'adsorption d'ions phosphates sur γ -FeOOH limite l'accès des électrons au FeIII de l'oxyhydroxyde, et est appelée « passivation » de cette surface. Cette passivation limite le transfert d'électrons au FeIII, qui se traduit par des cinétiques de production de FeII plus lentes qu'en leur absence (Borggaard et al., 2004). L'adsorption d'autres molécules stériques s'opposant à la mobilité des électrons affecte similairement la vitesse de réduction des oxydes métalliques.

La réduction des métaux n'engendre pas une mise en solution systématique. Par exemple, la réduction du FeIII structural des argiles (Boivin et al., 2002 ; Favre, 2002, Favre, 2004) engendre un changement de la capacité d'échange en cations (C.E.C.), lié à la quantité de Fe réduit, à la perte possible de groupements hydroxyls des argiles (Heller-Kallai, 1997), et à la réorganisation de la structure des argiles (Manceau et al., 2000). Par ailleurs, en fonction des conditions du milieu, les métaux en solution peuvent précipiter. Ces phénomènes de précipitation seront décrits plus précisément dans le paragraphe § II.2.2.5.

II.2.2.3 Les bactéries réductrices de FeIII et d'autres éléments métalliques

La grande biodiversité des bactéries dans l'environnement et la diversité des différentes activités associées à ces microorganismes jouent un rôle fondamental sur les cycles biogéochimiques et les flux de matières et d'énergie. Afin de mieux comprendre le rôle des microorganismes dans le fonctionnement des écosystèmes et les dynamiques de populations microbiennes en milieu complexe, différentes classifications ont été établies, telles que i) la classification taxonomique, regroupant les bactéries selon leur similitudes mutuelles, les taxons, ii) la classification phénotypique, basée sur des caractéristiques morphologiques, culturelles, métaboliques, la composition chimique, iii) la classification moléculaire basée sur l'analyse de l'architecture génomique globale, et iv) la typologie par groupes fonctionnels. Pour cette dernière classification, les groupes fonctionnels sont définis par la nature des accepteurs et des donneurs d'électrons impliqués dans le catabolisme. En anaérobiose, la classification des bactéries en groupes fonctionnels comprend souvent les dénitrifiants, les fermentaires, les réducteurs de SO_4^{2-} , les réducteurs des métaux, les acétogènes, les méthanogènes... Toutefois, il apparaît fréquemment qu'une bactérie ait la capacité de changer de métabolisme en réaction à des modifications de son environnement (stress, inhibitions, régulations) ou d'avoir plusieurs activités cataboliques simultanées. Par exemple face à une limitation en substrat, un microorganisme pourrait assurer sa survie en changeant de métabolisme sans toujours en tirer l'énergie nécessaire pour se développer.

La réduction microbienne d'éléments métalliques comme FeIII et MnIV est généralement supposée être la principale origine de leur mobilisation (Lovley, 1991). Il est par conséquent important de caractériser les fonctionnalités des acteurs microbiens de ces biotransformations

et de quantifier en nombre et/ou en masse ces acteurs. Plusieurs auteurs ont ainsi tenté des dénombrements des réducteurs de FeIII (Ghiorse, 1988 ; Haveman et Pedersen, 2002 ; Küsel et al., 2002 ; Weiss et al., 2003). Les résultats auxquels nous avons eu accès sont résumés dans le [tableau 4](#). Si la réduction des métaux est un processus important dans les sols et les sédiments, les évaluations quantitatives restent peu nombreuses, et effectuées trop rarement sur les sols, et plus fréquemment à d'autres milieux, ou dans des situations particulières de ces milieux. Leur portée reste donc faible, d'autant que les méthodes de dénombrement elles-mêmes peuvent être critiquées (Hill et al., 2000).

Les microorganismes capable de réduire le FeIII représente une grande diversité phylogénétique, comprenant aussi bien des Archaea que des bactéries. La plupart des microorganismes qui réduisent FeIII ont la capacité de réduire MnIV. De nombreux donneurs d'électrons peuvent être utilisés par les réducteurs des métaux, comme l'acétate et les autres acides gras volatils (Lovley et Phillips, 1988), les sucres, polyols et acides aminés (Coates et al., 1999), les composés mono-aromatiques tels que benzoate, phénol et toluène (Lovley, 1995), et dans de nombreux cas H₂ (Coppi et al., 2004; Lovley et Goodwin, 1988). Toutefois chaque espèce réductrice de FeIII se distingue par son affinité vis-à-vis des donneurs d'électrons (Lovley et al., 1989). En plus de la réduction du FeIII et du MnIV contenus dans les oxydes du sol, certaines espèces bactériennes réductrices de FeIII et/ou de MnIV peuvent croître avec d'autres accepteurs d'électrons UVI, CrVI, SeVI, PbII, TcVII et HgII (Lovley, 1995). Par exemple, *G. metallireducens* et *S. putrefaciens* peuvent croître par réduction de UVI en UIV (Lovley et al., 1991 ; Lovley et al., 1993). De même, quelques microorganismes réducteurs de FeIII peuvent réduire TcVII (Istok et al., 2004) ou CrVI (Lloyd, 2003), bien qu'à notre connaissance aucune croissance bactérienne n'ait été observée. *S. barnesii* peut réduire SeVI en Se⁰ et AsV en AsIII (Laverman et al., 1995). Des réducteurs de FeIII ont également présenté une capacité à réduire des composés non métalliques, tels que le fumarate en succinate (Coppi et al., 2004) , NO₃⁻ (Coates et al., 1989) et S⁰ en sulfure (Loneragan et al., 1996). Réciproquement beaucoup de bactéries fermentaires ont la possibilité d'utiliser FeIII et MnIV comme accepteurs d'électrons mineurs au cours de fermentation (Lovley, 1991). Cependant bien qu'une importante quantité de FeII et MnII puisse être produite par cette voie, la croissance bactérienne est alors très faible. De même un grand nombre de bactéries sulfato-réductrices peuvent également réduire FeIII (Coleman et al., 1993) ; A notre connaissance, une seule étude (Tebo et Obratzsova, 1998) aurait révélé une croissance des sulfato-réducteurs par la réduction de FeIII ou du MnIV. Ponctuellement, des méthanogènes, *Methanosarcina barkeri* MS et *Methanococcus voltaei* A3, peuvent réduire des quantités

Tableau 4: Dénombrements réducteurs FeIII.

| Microorganismes | Dénombrement | Milieu | Représentativité des Réducteurs de métaux | Références |
|---|---|---|--|---------------------------|
| Réducteurs FeIII (cellules.mL ⁻¹) | (1) 1.3 10 ¹ – 1.7 10 ⁴ | Solution du sol (3 stations) de prélèvement | Quantité totale de cellules: 10 ⁴ –10 ⁶ cellules.mL ⁻¹ | Haveman et Pedersen, 2002 |
| H ₂ -Réducteurs FeIII Acétate-Réducteurs FeIII (MPN.g ⁻¹ sol sec) | 3.2 10 ⁴ – 7.1 10 ⁵ 4.2 10 ⁴ – 9.3 10 ⁵ | Sol de prairie d'Hawiari (Parc national Kaena) Roches volcaniques, A horizon | | Küsel et al., 2002 |
| Réducteurs FeIII (racines) Réducteurs FeIII (Rhiz.) (cellules.g ⁻¹ sol sec) | 1.4 10 ⁹ 8.6 10 ⁷ | Rhizosphère de plantes en tourbières | 12 % des bactéries au niveau des racines et moins de 1 % au niveau de la rhizosphère | Weiss et al., 2003 |
| Réducteurs FeIII (cellules.mL ⁻¹) | 10 ⁵ –10 ⁶ | À l'interface eau-sable | | Sobolev and Roden, 2001 |
| Glucose-Réducteurs FeIII H ₂ -Réducteurs FeIII | 4.9 10 ² –1.9 10 ⁴ <3 | Sédiments acides Bactérie isolée: <i>Acidiphilium cryptum</i> JF-5 | Quantité totale de cellules: 7.2 10 ⁵ (5.8 10 ⁵ – 8.6 10 ⁵) (cellules.g ⁻¹ sol humide – coloration au DAPI) | Küsel et al., 1999 |
| Acétate-Réducteurs FeIII (cellules.g ⁻¹ sol humide) Lactate-Réducteurs FeIII (cellules.mL ⁻¹) | <3 0 – 0.5 cm: 0.07 (0.00); 1.5 – 2.5 cm: 0.56 (0.03) 6.5 – 7.5cm: <0.01 | Sédiment (eau douce) Lac constance A différentes profondeurs | Quantité totale de cellules: 10 ⁶ cellules sédiment.mL ⁻¹ | Kappler et al., 2003 |
| Acétate-Réducteurs FeIII (cellules.mL ⁻¹) | 0 – 0.5 cm: 0.08 (0.00) 1.5 – 2.5 cm: 0.47 (0.02) 6.5 – 7.5cm: 0.01 (0.00) | | | |
| Réducteurs Métaux (cellules.g ⁻¹ soil sec) | 0.6 10 ⁴ – 1.7 10 ⁴ | Concrétions de la mer Baltique | MRB vs hétérotrophes totaux 15–27 % (concrétions) | Ghiorse, 1988 |
| Acétate-Réducteur MnIV (cellules.mL ⁻¹) | 0.6 10 ⁴ – 2.5 10 ⁴ Station 1-3: 2.1 10 ³ ; 1.1 10 ³ Station 2: 1.1 10 ⁵ Station 4: 460 | Sédiments Sédiments de la Mer Noire | 25-36 % (sédiments) Niche de Réducteurs Mn IV Dénombrement réducteurs de FeIII: 23 cellules.mL ⁻¹ | Thandrup et al., 2000 |

significatives de FeIII en utilisant H₂ comme donneur d'électrons, et la présence d'AQDS ("navette d'électrons") a accéléré la réduction du FeIII pour ces deux microorganismes (Bond et Lovley, 2002).

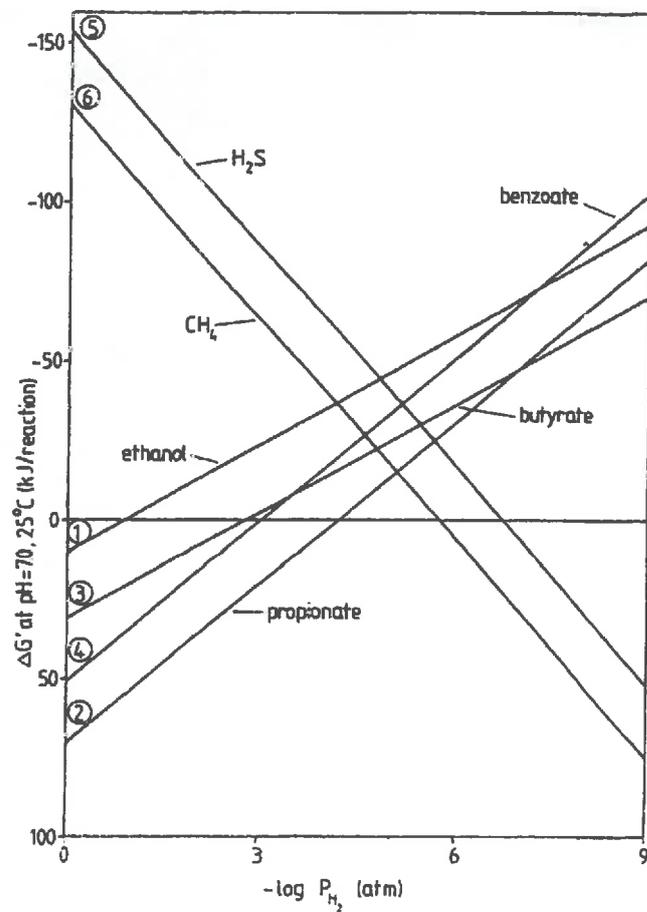
II.2.2.4 Effet des donneurs et des accepteurs d'électrons

Parce que la réduction des métaux fait souvent appel à des produits intermédiaires des voies cataboliques anaérobies (H₂ ou acides organiques volatils notamment), tous les processus affectant le devenir de la matière organique et celui de H₂ vont interférer avec celui des métaux (Chen et al., 2003).

En anaérobiose, la dégradation de la matière organique est un processus complexe. Dans un premier temps, les polymères glucidiques, protidiques et lipidiques sont hydrolysés sous forme de monomères, petits sucres, acides aminés, monomères lipidiques, acides organiques ... (Lovley, 1991). L'hydrolyse est souvent considérée comme la phase limitante des processus ultérieurs (McInerney, 1988). Ces monomères sont alors oxydés partiellement ou complètement par des fermentations (Colberg, 1988) ou des respirations diverses telles que la dénitrification (Tiedje, 1988) ou les respirations sur oxy-hydroxydes métalliques (Widdel, 1988). Les sucres et acides aminés peuvent être dégradés par voie fermentaire et aboutir à la production d'acides organiques, voire d'alcools. Les petites molécules issues de la dégradation fermentaire peuvent être reprises par les dénitrifiants, les réducteurs des métaux, certains SO₄²⁻-réducteurs (Widdel, 1988) ou acétogènes réducteurs de protons (Müller, 2003) pour aboutir suivant les cas à une minéralisation totale des éléments ou à la production d'acétate principalement. L'acétate peut être produit par homoacétogénèse à partir de CO₂ et H₂ (Diekert et Wohlfarth, 1994). Il sert aussi de substrats à la méthanogénèse acétoclaste (Fey et Conrad, 2000), CO₂ et H₂ étant de leur côté les substrats de la méthanogénèse hydrogénoclaste (Chin et Conrad, 1995).

A côté des monomères glucidiques et protidiques (Coates et al., 1999) et des acides organiques (Lovley et Phillips, 1988) produits et/ou consommés par voies microbiennes et dont la disponibilité dépend d'adsorptions/désorptions sur les phases solides (Loll et Bollag, 1986) ou de phénomènes d'absorption/excrétion, H₂ apparaît comme le donneur d'électrons

Figure 1: Effet de la pression partielle en H₂ sur l'énergie de différentes voies cataboliques utilisant H₂ comme substrat.



privilegié des réducteurs des métaux (Coppi et al., 2004). L'hydrogène est principalement produit par lyse du formiate et par acétogénèse réductrice de protons. Au contraire, la réduction initiale de NO_3^- en NO_2^- peut se faire avec oxydation du formiate, engendrant alors un défaut de production en H_2 par lyse du formiate. La réduction de SO_4^{2-} avec oxydation d'acides organiques ou d'alcools en acétate réduit également la production de H_2 par acétogénèse réductrice de protons. D'un autre côté, l'homoacétogénèse (Diekert et Wohlfarth, 1994), la fixation non-symbiotique de N (Pelmont, 1993), la méthanogénèse hydrogénoclaste (Oremland, 1988) et la réduction de SO_4^{2-} (Acht nich et al., 1995a) sont des voies métaboliques consommatrices de H_2 . La pression en H_2 régule de nombreuses activités microbiennes (Dolfing, 1998) (Fig.1). La réduction des métaux peut s'effectuer à une pression partielle en H_2 très faible. L'oxydation du H_2 concomitante à la réduction de FeIII et de MnIV peut ainsi être effectuée à des pressions partielles de H_2 plus faibles que les valeurs minimales nécessaires à la réduction de SO_4^{2-} et à la méthanogénèse hydrogénoclaste. *Shewanella putrefaciens* métabolise H_2 avec la réduction de FeIII à une pression en H_2 (0.007 Pa), 25 fois inférieure à la pression de H_2 minimale nécessaire à la réduction de SO_4^{2-} (Lovley et al., 1989). Les bactéries réductrices de FeIII peuvent ainsi maintenir la pression en H_2 en dessous des pressions seuil de la sulfatoréduction et de la méthanogénèse hydrogénoclaste (H_2 seuil : 1.0 – 5.0 Pa (Acht nich et al., 1995b)) bloquant ainsi ces voies métaboliques (Conrad, 1996).

De précédentes études ont montré un effet dépressif de NO_3^- sur les réductions de FeIII et de MnIV. Cependant, en milieu non limitant en donneurs d'électron communs, la diminution des réductions de FeIII et de MnIV par le NO_3^- endogène au sol est généralement limitée (Acht nich et al., 1995a). Cet effet dépressif peut être dû à la consommation d'un substrat organique commun, à une réoxydation abiotique de FeII en FeIII par NO_2^- produit au cours de la réduction de NO_3^- (Komatsu et al., 1978), ce qui pourrait alors masquer pour partie l'ampleur de la réduction de FeIII. L'accumulation de NO_2^- pourrait également avoir un effet toxique temporaire ou irréversible, sur les microorganismes. A notre connaissance, il n'a pas été reconnu d'effet dépressif de SO_4^{2-} sur la réduction du FeIII. Au contraire, FeII en solution peut augmenter par réaction abiotique la ré-oxydation de S^{2-} en SO_4^{2-} associée à la réduction de FeIII en FeII (Murase et Kimura, 1997).

Les oxydes métalliques présentent une réactivité très variable. Des expériences comparant la réduction microbienne par *Shewanella alga BrY* de FeIII amorphe, de goethite et d'hématite montrèrent que 20 fois plus d'oxyde de FeIII amorphe que de goethite et 50 fois plus de goethite que d'hématite ont été réduits lors d'une incubation de 5 jours (Roden et Zachara, 1996). En système de culture, la réduction microbienne induit souvent la formation de minéraux tels que la magnetite, la sidérite, la vivianite et la rhodocrosite, ainsi que des précipités de sulfures et carbonates. La formation de ces minéraux en milieu de culture explique d'importants dépôts de ces minéraux dans les sols.

II.2.2.5 Complexation et mobilité des métaux en anaérobiose

Dans la solution du sol, les métaux existent sous formes (i) libres ou ioniques, (ii) complexées avec des ligands inorganiques (Cl^- , CO_3^{2-} , NO_3^- ...) ou organiques (acétate, EDTA, MON...), ou adsorbés à la surface d'organismes vivants (microorganismes, poissons ...). Les métaux de transition (Cu, Zn, Ni, Cr, Au, Ag, Cd, Hg) sont particulièrement susceptibles de former des complexes avec des ligands variés (Arnaud, 1997) qui jouent un rôle important dans leur mobilité. Des études antérieures (Lovley et Woodward, 1996 ; Urrutia et al., 1999) ont montré que les agents chélateurs, tels que le NTA, l'EDTA, stimulaient la réduction des oxydes de FeIII, avec des cinétiques de réduction croissantes avec l'augmentation en concentration en chélateurs. Deux processus ont été mis en évidence (i) la mobilisation et complexation de FeIII de la surface des oxydes (Lovley et Woodward, 1995) et (ii) la complexation de FeII produit au cours de la réduction des oxydes (Urrutia et al., 1999 ; Roden et Urrutia, 1999). La formation de complexes avec FeII en solution conduit à une augmentation du FeII mobilisé. Un complexant moins fort, tel que le malate présente également la capacité de stimuler la réduction microbienne de la goethite (Urrutia et al., 1988), au même titre que les substances humiques et tous les acides organiques, acides aminés, en tant que ligands (Roden et Urrutia, 1999). Dans ces expériences, Urrutia et al. (1988) démontrèrent que la complexation avec le NTA se faisait aux dépens de l'adsorption sur la goethite. De même, les oxydes d'Al ont une capacité d'adsorption plus forte que la goethite par exemple ($6.9 \mu\text{mol FeII m}^{-2}$ et $4.4 \mu\text{mol FeII m}^{-2}$, respectivement) (Urrutia et al., 1988). La proximité d'oxydes d'Al avec des oxydes de FeIII stimule la réduction microbienne

de FeIII, suite à l'adsorption de FeII produit sur l'oxydes d'Al (Urrutia et al., 1988). La complexation par des ligands en solution ou sur d'autres phases solides stimule et prolonge la production de FeII.

La complexation entre un métal et un ligand est définie par la constante de complexation, représentant la force de liaison entre le métal et le ligand, et la capacité complexante du ligand, c'est-à-dire la quantité de métal qui peut se lier au ligand. L'impact de la complexation sur la mobilisation des éléments métalliques dépend donc de ces deux variables. Le [tableau 5](#) présente la constante de complexation pour différents complexes. La matière organique des sols est constituée de molécules organiques extrêmement variables par leur complexité et structure allant d'acides organiques, de sucres, protéines, acides aminés, alcools jusqu'aux acides humiques et fulviques. Même si certains métaux sont connus pour se fixer préférentiellement sur certains sites (cas du cuivre avec les groupes carboxyles ou bien le mercure pour les groupements sulfurés), la plupart des éléments entrent en compétition pour l'accès aux sites de complexation des matières organiques dissoutes. Une séquence de stabilité des complexes a été établie pour les sols par Irving Williams (1948), cité dans [Förstner \(1985\)](#) avec par ordre décroissant de stabilité $Pb > Cu > Ni > Co > Zn > Cd > Fe > Mn > Mg$. La spéciation des métaux est affectée par le *pH*, le potentiel d'oxydoréduction, la nature et la concentration en complexants dans la solution, les concentrations en ions « compétiteurs » ([Takacs et Alberts, 1999](#)), la température et les cinétiques de réaction. La spéciation des métaux varie ainsi dans le temps et l'espace. La dégradation de ligands peut aboutir à une augmentation de proportion sous forme libre, résultant soit à une mobilité accrue, soit à une immobilisation par précipitation ([Stumm et Morgan, 1996](#)).

Les métaux en solution peuvent précipiter ([Förstner and Wittmann, 1983](#)) sous formes d'oxyhydroxydes, de carbonates, de sulfures, de phosphates ([Stumm et Morgan, 1996](#) ; [Pankow, 1991](#)) pour les plus courants ou de précipités mixtes ([Banfield et Hamers, 1997](#) ; [Webster et al., 1998](#)). La magnétite ([Frederickson et al., 1998](#)), la sidérite ([Stumm et Morgan, 1996](#)), la vivianite ([Dong et al., 2000](#)), la goethite ([Glasauer et al., 2003](#)) et les rouilles vertes ([Ona N'Guéma, 2003](#)) sont des minéraux communs produits au cours de réduction de FeIII. Les métaux passent alors en phase solide et deviennent moins facilement solubles. Selon la définition de [Sposito \(1986\)](#), le mécanisme de précipitation est initié par une nucléation homogène ou hétérogène. Elle peut ainsi engendrer un solide mixte par inclusion ou par coprécipitation ([Crawford et al., 1996](#)), ou s'effectuer à la surface d'une phase solide

Tableau 5: Exemple de constantes de complexation (Parker et al., 1995).

| Ligands | Constante de complexation | | | | | |
|-------------------------------|---------------------------|-------|-------|-------|-------|-------|
| | FeII | MnII | CuII | ZnII | PbII | CrIII |
| EDTA | 19.3 | 19.1 | 24.0 | 21.7 | 23.0 | 29.2 |
| NTA | 13.6 | 11.6 | 18.2 | 14.9 | 12.7 | - |
| Acétate | 1.4 | 2.2 | 3.5 | 2.9 | 4.1 | - |
| Citrate | 10.1 | 9.2 | 10.8 | 9.4 | 10.1 | - |
| Oxalate | - | 5.3 | 10.0 | 7.3 | 0 | 6.4 |
| Cystéine | 4.3 | 6.7 | 16.0 | 9.7 | 10.6 | - |
| Glutamate | 5.0 | 4.3 | 15.5 | 10.2 | - | - |
| Subs. Fulviques (1) | 4.4 | 4.4 | 4.3 | 4.0 | 4.7 | - |
| Subs. Fulviques (2) | 2.7 | 2.7 | 2.5 | 2.2 | 4.0 | |
| CO ₃ ²⁻ | 0.0 | 4.5 | 6.7 | 4.8 | 0.0 | 13.5 |
| OH ⁻ | -20.6 | -22.2 | -13.8 | -16.8 | -17.8 | -9.7 |
| SO ₄ ²⁻ | 2.2 | 2.3 | 2.4 | 2.3 | 2.6 | 3.2 |

préexistante. Le phénomène de précipitation dépend du pH, des concentrations en composés organiques et en métaux, représenté par le rapport des concentrations en métal et en ligands (Stumm et Morgan, 1996), de la force ionique de la solution, de la taille des particules, de la réactivité de la surface des phases solides, en relation avec la présence de molécules adsorbées ou la formation de biofilms (Brown et al., 1999a, b), et finalement de la vitesse de réduction du métal (Ona Nguéma, 2003). Bien que les processus de nucléation et de croissance soient identiques par voie abiotique et biologique, la formation de minéraux en présence de bactéries réductrices de FeIII dépend de la vitesse de réduction du FeII (Ona Nguéma, 2003). La capacité des bactéries à réduire FeIII affecte la nature du minéral formé et sa vitesse de cristallisation (Roden et Zachara, 1996 ; Zachara et al., 1998; Liu et al., 2001). Ona N'Guema (2003) observa également la formation de sidérite en partant de goethite. De manière similaire dans la formation de rouilles vertes, la vitesse initiale de production de FeII est le facteur qui contrôle la formation de rouilles vertes au détriment de la magnétite (Ona Nguéma, 2003).

II.2.3 Evolution ultérieure aérobie

L'anoxie n'est généralement qu'un état transitoire, souvent limité à des zones d'extensions spatiales réduites (Brune et al., 2000). Il est plus généralement observé des alternances dans le temps ou des juxtapositions spatiales de conditions anaérobies et aérobies (Öztürk, 1995 ; Granina et al., 2004). C'est le cas dans le sol (i) au sein de la rhizosphère (Weiss et al., 2003 ; Bidel et al., 2000), (ii) au cœur des mottes ou agrégats (Sierra et al., 1995), et (iii) au voisinage de matières organiques en décomposition (Parry et al., 2000). C'est encore vrai pour des conditions de milieu plus extrêmes comme les rizières (Boivin et al., 2002 ; Liesack et al., 2000 ; Lüdemann et al., 2000), et les sédiments (Oguz et al., 2001).

Ces alternances entre anaérobiose et aérobiose soulèvent plusieurs interrogations relatives à :

- la résilience du système après retour à l'aérobiose (minéralogie, biodiversité et fonctionnalité de la microflore), et la caractérisation des échelles de temps des transformations aérobies ;
- l'impact de la coexistence de composés oxydés et réduits (rouilles vertes et NO_3^- , NO_2^- et NH_4^+ ... par exemple) sur les évolutions biogéochimiques ;

- le maintien ou non du "potentiel microbien" de biotransformations anaérobies après un épisode aérobie.

A notre connaissance, les transformations biogéochimiques et la résilience du système après retour à l'aérobiose restent peu étudiées (van Bodegom et al., 2001).

Concernant les oxydations de FeII et MnII, elles peuvent s'effectuer par voie biologique ou abiotique (Tebo et He, 1999; Frankel et Bazilinsky, 2003). En condition aérée et à un pH>5, l'oxydation abiotique de FeII en solution est rapide avec une demi vie de 2 à 10 min (Stumm et Morgan, 1996). Ainsi, l'oxydation microbienne de FeII en condition aérobie à pH>5 peut être perçue comme un processus mineur n'ayant pas réellement la possibilité de s'exprimer (Emerson et Weiss, 2004; Frankel et Bazilinsky, 2003). La vitesse d'oxydation abiotique de FeII diminue avec la concentration en O₂ pour des teneurs inférieures à 10 % en phase gazeuse. A notre connaissance, aucune bactérie ne bénéficiant d'énergie via l'oxydation de FeII n'a été jusqu'à présent isolée et étudiée à pH neutre et en conditions aérobies (Emerson et Revsbech, 1994). Ainsi la croissance des bactéries neutrophiles oxydantes de FeII a plus spécifiquement été observée dans des environnements microaérobies, où les concentrations en O₂ sont basses. A des teneurs de 0.5 < O₂ < 3.0 %, l'oxydation microbienne représente 45 – 80 % (Emerson et Revsbech, 1994), 50 – 60 % (sans atteindre une croissance maximale sur milieu de culture) (Neubauer et 2002) et 90 % (Sobolev et Roden, 2001) de l'oxydation totale de FeII. Les constantes d'oxydation avec ou non la différenciation entre activité microbienne et oxydation abiotique sont référées dans le [tableau 6](#). Au contraire en milieu acide, de nombreuses bactéries acidophiles oxydantes de FeII ont été isolées, comprenant *Thiobacillus ferrooxidans*, *Leptospirillum* sp. *Sulfobacillus* spp., *Acidimicrobium ferrooxidans*...(Frankel et Bazilinsky, 2003).

A l'inverse de FeII, l'oxydation abiotique de MnII en condition aérobie est une réaction très lente ([Tableau 6](#)), qui n'a été observée qu'à des pH supérieurs à 8 (Morgan et Davies, 1989). La demi vie de MnII à pH 8 est d'environ 400 j (Morgan, 2005). A notre connaissance, l'oxydation abiotique de MnII n'a jusqu'à présent pas été observée à pH neutre (Morgan, 2005). Par conséquent à pH inférieur à 8 (cas de très nombreux sols) et en conditions de bonne aération, l'oxydation de MnII se ferait préférentiellement par voie microbienne. L'oxydation biologique de MnII est généralement menée par des microorganismes qui effectuent également l'oxydation biologique de FeII (Tebo et He, 1999), à l'exception des bactéries de forme "filamenteuse" du genre *Gallionella*. En particulier, l'oxydation de MnII

Tableau 7: Constantes des oxydations de FeII et MnII.

| Element oxydé | Milieu | Constante d'oxydation s^{-1} | Contribution microbienne | Contribution abiotique | pH | O ₂ | Température | Références |
|---------------|-------------------------------------|--------------------------------|--------------------------|------------------------|------|------------------------------|-------------|---------------------------------|
| FeII | Sol de rizière, séché à l'air Batch | $1.74 \cdot 10^{-4}$ | | principale | | 20 % | 30 °C | Van Bodegom et al., 2001 |
| FeII | Sol | - | > 50 – 60 % | < 40 – 50 % | | 0.5 < O ₂ < 3.0 % | | Neubauer et al., 2002 |
| FeII | Sol | - | 45 – 80 % | 20 – 55 % | | 0.5 < O ₂ < 3.0 % | | Emerson et Revsbech, 1994 |
| FeII | Sol | - | 90 % | | | 0.5 < O ₂ < 3.0 % | | Sobolev et Roden, 2001 |
| FeII | Réacteurs | $1.17 \cdot 10^{-2}$ | 50 % | 50 % | 7.2 | D.O.: 119-62.5 μ M | | Katsoyiannis et Zouboulis, 2004 |
| MnII | Eau de mer | $2.31 \cdot 10^{-8}$ | | | 8.00 | 0.22 atm | 25°C | Von Langen et al., 1997 |
| | | $3.14 \cdot 10^{-8}$ | | | 8.17 | (D.O.: 215 μ M) | | |
| | | $6.02 \cdot 10^{-8}$ | | principale | 8.45 | | | |
| | | $1.03 \cdot 10^{-7}$ | faible | | 8.65 | | | |
| MnII | | $< 3.38 \cdot 10^{-8}$ | | | 8.35 | | | Morgan et Davies, 1989 |
| | | $1.50 \cdot 10^{-6}$ | | | 8.95 | | | |
| | Goethite | $3.00 \cdot 10^{-4}$ | | 100 % | 8.03 | | | |
| | Ferrihydrite | $3.00 \cdot 10^{-4}$ | | | 8.03 | | | |
| MnII | Réacteurs | $2.90 \cdot 10^{-3}$ | principale | | 7.2 | D.O.: 119-62.5 μ M | | Katsoyiannis et Zouboulis, 2004 |

peut être effectuée par des espèces de différents genres, tels *Leptothrix*, *Crenothrix*, *Hyphomicrobium*, *Siderocapsa* et *Metallogenium*. Pour une solution du sol en équilibre avec une atmosphère à 10 % d'O₂ environ et à pH 7.2 (température non précisée), puis inoculée avec *Leptothrix ochracea* et *Gallionella ferruginea*, [Katsoyiannis et Zouboulous \(2004\)](#) établirent que l'ensemble de l'oxydation de MnII était microbienne avec une constante d'oxydation 2000 fois supérieure à l'oxydation abiotique, ($k = 2.90 \cdot 10^{-3} \text{ s}^{-1}$). De même Morgan proposa une demi vie pour l'oxydation du MnII à pH 8 de 400 j par voie abiotique et 10 H par voie biologique.

Les oxydations de FeII et MnII sont affectées par diverses variables :

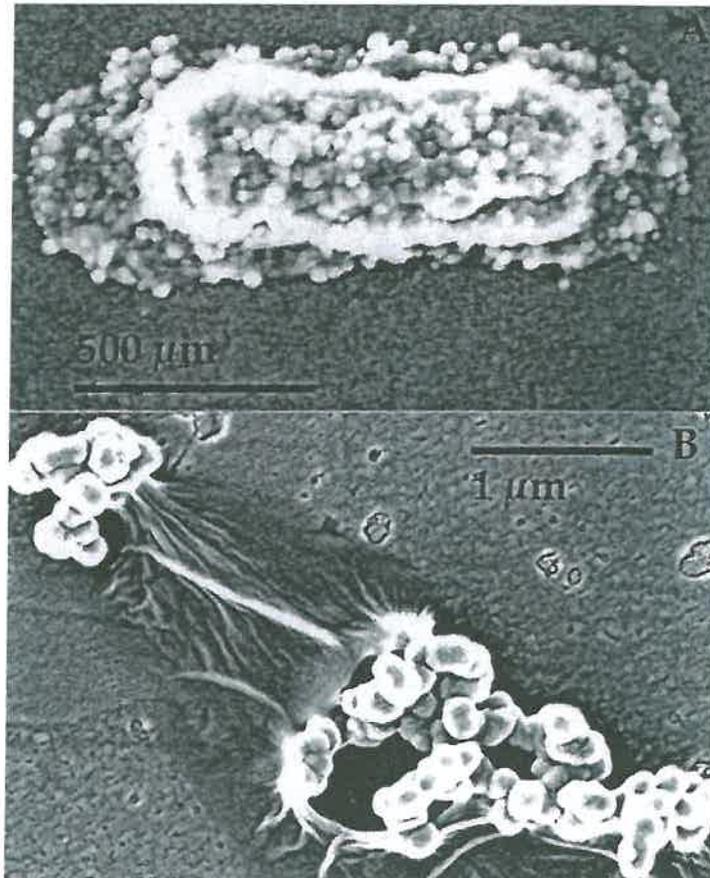
- le pH, la vitesse d'oxydation du FeII par voie abiotique étant multipliée par 100 pour une augmentation du pH d'une unité et pour des pH supérieurs à 5 ([Tebo et He, 1999](#)). De même, la constante d'oxydation du MnII par voie abiotique est 60 fois plus importante par l'augmentation d'une unité de pH pour des pH supérieurs à 8 ([Tableau 7](#)) ([Jun et Martin, 2003](#)). Les activités microbiennes sont également affectées par le pH ;
- les surfaces catalysant l'oxydation de FeII et MnII : oxydes de MnIV et de FeIII, ainsi que les parois des cellules bactériennes ([Morgan, 2005](#) ; [Morgan et Davies, 1989](#)). Ainsi, le [tableau 7](#) présente des cinétiques d'oxydation de FeII plus rapides d'un facteur 20 en présence de goethite et de ferrihydrite en comparaison de réactions abiotiques sans catalyseur. De même, l'oxydation de MnII en condition aqueuse est lente initialement ; une fois activée, elle se poursuit avec une oxydation hétérogène de plus en plus rapide et avec la production d'oxydes de MnIV particulière. [Morgan \(2005\)](#) proposa une demi vie pour l'oxydation de MnII à pH 8, de 30 j en présence de goethite ou ferrihydrite (catalyseur de surface), et de 400 j par voie abiotique ;
- les complexations de FeII et MnII avec des ligands minéraux et organiques ou également des molécules organiques extracellulaires, de type exopolymères, excrétées par les bactéries ([Neubauer et al., 2002](#)). qui ralentissent les cinétiques d'oxydation de FeII et MnII ([Millero et al., 1987](#)), et ce en fonction de la stabilité des complexations engagées et de l'évolution des ligands lors d'un retour à l'aérobiose. En aérobiose l'oxydation de ces derniers peut être soit microbienne exclusivement (NH₃, composés organiques ([van Bodegom, 2001](#))), ou microbienne et abiotique (sulfures). Le cas des

carbonates est particulier car lié à la phase gazeuse et à la diffusion de CO₂ en dehors du sol.

Lors d'un retour à l'aérobiose, l'oxydation de FeII et de MnII peut aboutir à la formation d'oxydes par voie microbienne ou par voie abiotique (Tebo et He, 1999). La ferrihydrite se forme par oxydation abiotique ou biotique de FeII dissous suivi d'une précipitation de FeIII. Ce minéral, le plus instable des oxydes de FeIII, se transforme ensuite (i) soit en goethite spontanément, (ii) soit en hématite, par réarrangement structural et déshydratation de la ferrihydrite. L'oxydation de MnII en solution ou adsorbé conduit à la formation de minéraux MnIII / MnIV, peu solubles. L'oxydation abiotique du MnII débute par la production d'oxydes de MnIII (e.g. Mn₃O₄) ou d'hydroxydes (MnOOH), qui se transforment lentement en oxydes de MnIV (e.g. MnO₂). L'oxydation des hydroxydes de MnII forme en milieu alcalin de la buserite qui, après déshydratation, aboutit à la birnessite qui, à son tour, évolue ensuite en pyrolusite.

Le changement d'aération favorise la dissolution des phases solides contenant FeII ou MnII, tels que les carbonates (Duckworth and Martin, 2004), les sulfures (Neal et al., 2001) et les oxyhydroxydes (Tebo and He, 1999). La nature des minéraux formés à la suite des réoxydations dépend de la vitesse de dissolution des minéraux de FeII et MnII. En conditions aérobies, la dissolution abiotique de FeCO₃ s'effectue à une vitesse de 10⁻¹⁰ mol m⁻² s⁻¹ pour un pH dans la gamme de 6.0 à 10.3 (Duckworth et Martin, 2004) et l'oxydation de FeII aboutit alors à la formation de ferrihydrite. De même, la dissolution de MnCO₃ s'effectue (i) à une vitesse de 10^{-8.45} mol m⁻² s⁻¹ (Duckworth et Martin, 2004) avec la formation successive de bixbyite pour un pH entre 5.8 et 7.7, (ii) à une vitesse de dissolution abiotique de 10^{-9.0} mol m⁻² s⁻¹ pour un pH supérieur à 7, engendrant la formation de bixbyite (5.8 < pH < 7.0) et feitnkechtite (pH > 7.7). (Duckworth et Martin, 2004). L'exposition à O₂ engendre l'oxydation des sulfures par voie abiotique (Poulton et al., 2004) ou biotique (Edwards et al., 2000). Elle est observée plus particulièrement dans les sols devenus très acides, tels que les sols miniers, suite à une exposition à l'air et à l'eau (Rimstidt et Vaughan, 2003). A faible pH, la vitesse de dissolution par oxydation dépend des concentrations en O₂ et en FeIII : FeIII peut être l'oxydant principal de la pyrite, avec laquelle il interagit plus efficacement que O₂ (McKibben et Barnes, 1986; Edwards et al., 2001). La minéralisation des sulfures est facilitée par des bactéries, qui peuvent oxyder les sulfures de FeII. La plus étudiée de ces bactéries oxydantes des sulfures est l'*Acidithiobacillus ferrooxidans*, bactérie acidophile autotrophe.

Figure 2: SEM de la formation d'oxyhydroxydes à la suite de l'oxydation microbienne par l'*Acidithiobacillus ferroxidans* (A), formation d'oxyhydroxydes ferriques dans des exopolymères microbiens (B) (Frankel et Bazilinsky, 2003).



Les précipitations ultérieures à l'oxydation de sulfures comportent de nombreux minéraux, tels que la ferrihydrite et la goethite, la jarosite et la schwertmanite (Frankel et Bazylinski, 2003). Finalement l'élément sulfur peut être également produit. Ces phénomènes de dissolution / précipitation ont un impact sur les cycles du Fe et du Mn mais également des autres métaux (U, Co, Pb, Ni, Cu et Zn), qui co-précipitent avec les oxydes de fer et de manganèse (Hem, 1989 ; Duff et al., 2002).

II.3 Objectifs de la thèse

Des problèmes croissants de gestion des déchets à La Réunion induisent une forte demande d'expertise et de recherche de la part des institutions locales (Chambre d'Agriculture) ou engagées dans la gestion du patrimoine environnemental (ADEME, CIRAD, INRA). L'objectif de ce travail était de contribuer à l'évaluation des risques de mobilisation d'éléments métalliques préexistants dans les sols (Fe, Mn ...) suite à un apport de déchets liquides chargés en composés fermentescibles. Le temps imparti à cette étude nous a conduit à nous focaliser sur l'étude de la réactivité biogéochimique. Au travers de cette synthèse bibliographique, nous avons fait le point sur les connaissances actuelles sur ce sujet et avons tenté d'identifier aussi quelques manques de connaissances.

Le 1^{er} objectif était de travailler à la hiérarchisation des besoins de description des grandes voies du catabolisme anaérobie des composés organiques (en fonction de la nature des apports organiques) pour définir leur contribution à la mobilisation des métaux. En effet, très peu de travaux décrivent les interactions entre transformations microbiennes et réactivité géochimique abiotique dans le sol : si Dassonville et al. (2004a) ont proposé un modèle couplant activités microbiennes anaérobies et géochimie abiotique, ce modèle n'est adapté qu'à des apports de glucose et NO_3^- , alors que la plupart des effluents liquides organiques sont des mélanges complexes de matières organiques (sucres, protéines, lipides, acides organiques, acides aminés, phénols...).

Le 2nd objectif était de mieux décrire ces interactions en couplant des suivis de dynamiques des réducteurs du FeIII (avec la prise en compte de leur aptitude à utiliser d'autres voies cataboliques) et d'autres communautés fonctionnelles dans un sol amendé avec

de la vinasse à des suivis relatifs à la réduction des métaux (FeIII et MnIV) et à la mobilisation de leurs formes réduites (FeII et MnII). A ce jour, les interactions entre dynamiques et fonctionnement(s) des réducteurs de FeIII et la réduction du FeIII restent encore trop peu connues, avec notamment les contributions relatives des réactions abiotiques et biologiques à la réduction de FeIII et MnIV (Murase et Kimura, 1997), les capacités des réducteurs du FeIII à d'autres métabolismes et par conséquent la contribution de ces autres métabolismes à la croissance des réducteurs de FeIII, et enfin l'utilisation de H₂ comme donneur d'électrons préférentiel ou non à la respiration des métaux.

Le 3^{ème} objectif était d'étudier l'oxydation de FeII, sa mobilisation à partir des phases solides et l'immobilisation de FeIII produit par oxydation suite à un retour à des conditions aérobies d'échantillons de sols préalablement incubés en anaérobiose. Plus concrètement, il s'agissait d'associer des échelles de temps aux évolutions aérobies de FeII et MnII, de suivre l'évolution de leurs principaux complexants minéraux et organiques, et d'appréhender sommairement l'évolution des phases solides contenant du Fe ainsi que de la biodiversité microbienne. Nos expériences n'ont pu dans le temps imparti répondre à l'impact du passage d'anaérobiose à l'aérobiose sur les bactéries du sol et leurs activités. En effet, la littérature ne donne accès qu'à peu de travaux traitant des vitesses d'oxydation pour FeII et MnII en fonction de leur caractère abiotique ou biologique, du pH, de la complexation de ces éléments métalliques et de la dissolution des minéraux contenant FeII et MnII.

III Possibilités d'extension à des apports de composés organiques divers d'un modèle biogéochimique développé pour des apports de cellulose, glucose et nitrate

L'épandage d'effluents riches en matières organiques a tendance à favoriser l'installation de conditions anaérobies. En l'absence d'O₂ et à la suite d'un apport riche en matière organique, les activités microbiennes engendrent des risques de réduction et mobilisation de certains métaux. Bien qu'étant généralement d'abord microbienne, la réduction des métaux, peut aussi s'effectuer par voie abiotique. Ainsi, les activités microbiennes affectent directement et indirectement le devenir des métaux par des changements de la solution (évolution du pH et du niveau d'oxydation, mobilisation de métaux, production de ligands minéraux et organiques) et des phases solides du sol (oxydoréductions biologiques et abiotiques d'éléments métalliques, dissolution et néoformations de minéraux). A ce jour, il existe peu de modèles combinant microbiologie et géochimie dans le sol. Ces modèles ont de plus été proposés initialement pour d'autres environnements (aquifère, digesteur anaérobie, rumen) et étaient soit à dominante microbienne sans tenir compte des évolutions géochimiques et des phases solides du sol, soit à dominante géochimique en décrivant les activités microbiennes de manière très sommaire. Dassonville et al. (2004) ont proposé le modèle Biogechem couplant biotransformations et réactivité géochimique en condition anaérobie, mais ce modèle a été développé pour des apports simples (cellulose, glucose, NO₃⁻), alors que la plupart des effluents liquides organiques sont des mélanges complexes de matières organiques (sucres, protéines, lipides, acides organiques, acides aminés, phénols...). L'objectif du travail, reporté dans le chapitre qui suit, était donc d'évaluer les possibilités d'extension de ce dernier modèle à des apports complexes en composés organiques.

Les expériences ont été basées sur des incubations anaérobies en batch à 25°C d'un Ferralsol (classification FAO) de l'île de La Réunion amendé avec de la vinasse de rhumerie. Trois traitements ont été effectués : (C) sol + eau, (+S+W) sol stérile + vinasse stérile diluée au 10^{ème}, et (+W) sol + vinasse diluée au 10^{ème}. Les incubations ont duré respectivement 40, 2

et 40 j pour les traitements (C), (+S+W) et (+W). Au cours de ces incubations, des mesures ont été effectuées sur les pools organiques de la solution du sol, sur des indicateurs d'activités microbiennes (gaz, solutés organiques et minéraux), les concentrations en métaux mobilisés (Fe, Mn, Cr, Ni), E_H et pH . Les traitements des données ont consisté à (i) caractériser la diversité des composés organiques, (ii) identifier les principales voies de dégradation et (iii) évaluer leurs effets sur la mobilisation des métaux.

Après 0.5 h d'incubation, le carbone organique dissous dans le traitement (C) représentait moins de 1.3 % de sa teneur dans les traitements avec apport de vinasse. A cette date, 79.5, 103.6, 38.5, 123.4, 62.1 et 17.4% respectivement des sucres, polyols, acides organiques, alcools, phénols et protides apportés étaient mesurés dans le traitement (+S+W). A 0.5 hr d'incubation, aucune différence importante d'immobilisation/mobilisation de ces pools n'a été observée entre les traitements (+S+W) et (+W) à l'exception des alcools, bien que le comportement des petits composés différaient entre traitements. Dans le traitement (+W), les dégradations des polyols, glucides, protides et acides organiques ont été simultanées. Elles peuvent être décrites par quelques réactions cataboliques sans qu'il soit nécessaire de tenir compte de la spécificité des composés appartenant à chacune de ces familles. Liées à la nature de nos apports, les fermentations, suivies de l'acétogénèse réductrice de protons, étaient largement prépondérantes en début d'incubation (de 0 à 21j) ; ultérieurement à partir de 21 j, la méthanogénèse est apparue. La réduction de FeIII et MnIV s'est effectuée dès le début de l'incubation jusqu'à 21 j, principalement avec l'oxydation concomitante de H_2 produit au cours de ces fermentations et de l'acétogénèse réductrice de protons, voire avec certains précurseurs de H_2 .

Ce chapitre 3 correspond à un article soumis à la revue *Soil Biology and Biochemistry* sous le titre, "Relative contributions of dissolved organic compounds supplied with rum vinasse to metal mobilisation in an anaerobic soil slurry", avec pour auteurs; N. Pautremat, P. Renault, P. Cazevielle, and S. Marlet.

Relative contributions of dissolved organic compounds supplied with rum vinasse to metal mobilisation in an anaerobic soil slurry

Abstract

Spreading liquid organic by-products of food industries on soil increases the risk of anaerobic mobilisation of metals. In order to assess the impact of rum vinasse on metal mobilisation in a Ferralsol (FAO classification) of Reunion Island, we performed anaerobic batch incubations at 25°C according to three treatments: (C) soil and water, (+S+W) sterilised soil and sterilised diluted vinasse, and (+W) soil and diluted vinasse. Incubations ended after 40, 2 and 40 d for treatments (C), (+S+W) and (+W), respectively. Along the incubation, we measured organic pools in solution (total carbon, carbohydrates, polyols, proteins, small acids, alcohols and phenols), indicators of microbial activities (gases, organic and mineral solutes), mobilised metal concentrations (Fe, Mn, Cr, Ni), E_H and pH . Data analyses aimed to (i) characterise the diversity of organic compounds, (ii) identify the main pathways for their degradation, and (iii) assess their effects on metal mobilisation. At 0.5 h, organic carbon in solution in treatment (C) represented less than 1.3% its value in vinasse treatments. At this time, 79.5, 103.6, 38.5, 123.4, 62.1 and 17.4% of carbohydrates, polyols, organic acids, alcohols, phenols and proteins, respectively, were recovered in solution of treatment (+S+W). At this date, no large deviation was observed between treatments (+S+W) and (+W) in the immobilisation/mobilisation of pools (except for alcohols), whereas behaviour of each compound differed between treatments. During incubation of treatment (+W), butyric and acetic fermentations, true acetogenesis and methanogenesis explained most of organic compound degradation. In contrast, denitrification was neglected due to the low initial NO_3^- content and SO_4^{2-} reduction could have either oxidised only about 15% of H_2 produced by fermentations and acetogenesis, or explained the lack of the equivalent production during butyrate or ethanol oxidation into acetate. Carbon balance indicated that most of carbohydrates, polyols, proteins and organic acids were used for these metabolisms and the related biomass increase. FeIII and MnIV reductions would thus have been reduced with H_2 oxidation, their mobilised forms representing less than 16% of the reduced quantities. Geochemical modelling indicated that siderite and rhodocrosite precipitations could explain this value. Complexed FeII and MnII represented less than 19 and 44.3%, respectively, of mobilised forms. In contrast most of Cr and Ni were mobilised since the beginning of incubation. Cr was mostly complexed with butyrate then with CO_3^{2-} and free Ni represented about 60 - 80% of mobilised Ni in treatment (+W).

III.1 Introduction

Spreading organic residues on soils allows their elimination and the soil enrichment with nutrient elements and organic matter (European Commission, 2001). This practice has been applied to liquid organic by-products of food industries, e.g. olive mill waste water (Zenjary and Nejmeddine, 2001), yeast factory wastes (Genç et al., 2002), fermentable wastes of distillery (Gemtos et al., 1999) or brewery wastes (European Commission, 2001), and wastes from dairy processing (Shi et al., 1999). The concomitant supply of water and organic compounds decreases gas diffusion (Collin and Rasmuson, 1988) and increases O_2 consumption rate, thus enhancing the risk of anaerobic conditions (Quantin et al., 2001). Anaerobiosis can have positive effects, including the elimination of NO_3^- (Khalil et al., 2005), chlorinated pesticides (Liu et al., 1991) and phenolic compounds (Lovley, 1991). However, it more usually has undesirable effects, including the emission of N_2O and CH_4 greenhouse gases (Conrad, 1996), the production and accumulation of toxic compounds (e.g. NO_2^- (Roy and Conrad, 1999), fatty acids (Aguilar et al., 1995), and C_2H_4 (Visser et al., 1997)), and the mobilisation of metals (Quantin et al., 2001).

In anaerobic condition, metal mobilisation occurs mainly by direct microbial reduction of metals contained in solid oxy-hydroxides (Lovley et al., 1989) and clays (Favre et al., 2002), although there are some efficient abiotic transformations, including the oxidation of sulfide into S^0 coupled with the reduction of FeIII into FeII (Murase and Kimura, 1997). Metal reducers involve a great functional diversity of bacteria (Straub et al., 2001), including communities that are also denitrifiers, SO_4^{2-} reducers or fermenters (Lovley, 1991). Metal reduction requires either bacteria contact with the solids (Das and Caccavo, 2000), or electron shuttle such as quinones (Nevin and Lovley, 2002), or complexation or chelation of metals enhancing their mobilisation (Kendall and Hochella, 2003). Microbial reduction of metals depends on concentration and nature of electron donors that include carbohydrates, polyols and protein monomers, fatty acids and H_2 (Lovley et al., 1989).

The net productions of monomers, fatty acids and H_2 result from various catabolic pathways (Dassonville and Renault, 2002). After the hydrolysis of polymers, monomers can be totally or partly oxidised by denitrification (Bremner, 1997), dissimilatory reduction of NO_3^- into NH_4^+ (Stouthamer, 1988), metal respirations (Lovley, 1991), and fermentations (Chidthaisong, 1999). Fermentation produces volatile fatty acids (e.g. formate, acetate, propionate, butyrate) and alcohols (e.g. ethanol) (Loll and Bollag, 1986), which can be used

for metal (Pohlman and Mc Coll, 1988) and SO_4^{2-} reductions (Widdel, 1988), acetogenic transformations (Dolfing, 1988), and acetoclastic methanogenesis (Vogels et al., 1988). H_2 is mainly produced through formate lysis (Lovley et al., 1989) and true acetogenic reactions (Dolfing, 1988); it is consumed through SO_4^{2-} (Widdel, 1988) and metal reductions (Ghiorse, 1988), homoacetogenesis (Dolfing, 1988), and hydrogenoclastic methanogenesis (Conrad, 1999). Anabolic activities also contribute to the consumption of up to 60% of organic compounds through anaerobic respirations such as denitrification (Stouthamer, 1988) and 10 to 20% through fermentations (Dassonville et al., 2004). Consumption of organic compounds may be slowed down by their adsorption on solid surfaces (Mayer and Xing, 2001), according to the order lipids > proteins > carbohydrates (Marschner and Kalbitz, 2003).

To describe metal mobilisation in anaerobic conditions, models have to distinguish microbial and abiotic geochemical transformations (Dassonville and Renault, 2002), since microbial and abiotic reductions/oxidations generally do not reach thermodynamic equilibrium (Stumm and Morgan, 1996), and microbial activities depend on factors differing from those affecting abiotic reactions (Zhender and Stumm, 1988). However, only a few models have tempted to combine microbial and abiotic geochemical transformations (Hunter et al., 1998; Vavilin et al., 2000; Dassonville et al., 2004). The model scarcity results from the diversity of involved transformations and their interactions (Dassonville et al., 2004). Especially, microbial activities drive most of soil geochemical changes in anaerobic conditions (Lovley, 1991) through reductions (Peter and Conrad, 1996), direct proton consumption or release, and indirectly induced pH changes (Stumm and Morgan, 1996), productions and consumptions of mineral and organic ligands that can increase metal mobilisation (Cambier and Charlatchka, 1999), as well as direct and indirect solid alterations (Lovley et al., 1991). Limits of existing models result from a simplified description of either microbial processes (Hunter et al., 1998), geochemical processes (Vavilin et al., 2000), or organic matter inputs (Dassonville et al., 2004). So far models have been developed on the basis of one or few organic molecules: e.g. glucose, formate, acetate, ethanol and butyrate for Dassonville et al. (2004), and carbohydrates, lipids, proteins, acetate, butyrate and propionate for Vavilin et al. (2000). In contrast, actual wastes contain a mixture of various carbohydrates, proteins, lipids, organic acids and phenols (Calamy and Moletta, 2002).

The aims of this work were to identify the main anaerobic microbial processes implied in the mobilisation of metals after supplying liquid organic waste, and link these processes to the nature of organic inputs and their biotransformations. The study was carried out in batch

conditions, using a Ferralsol (FAO classification) from the Reunion Island with high contents in Fe, Mn, Cr and Ni, and a vinasse supplied by a rum factory.

III.2 Materials and Methods

III.2.1 Soil and organic liquid waste

Experiments were performed on a Ferralsol sampled in Reunion Island at "La Mare" experimental station, 20°40' S and 55°32' E. Since 20 years, this experimental field has been used for sugarcane crop. The annual average precipitation is about 1500 mm with a potential evapotranspiration of 2300 mm. Sampling was performed on the 0-20 cm layer of the soil on the 3rd December 2002 after the harvest. The soil was then air-dried (9.67% residual moisture), stored in hermetic bags, and flown to Montpellier under cold conditions. It was mechanically crushed, sieved at 2 mm, and stored again in plastic bags at 4°C until the beginning of the experiments. Its properties were as follows: 679 g kg⁻¹ clay; 278 g kg⁻¹ silt; 44 g kg⁻¹ sand; 19.9 g kg⁻¹ organic C; 1.59 g kg⁻¹ organic N; 1.74 mg kg⁻¹ N-NH₄⁺; 6.16 mg kg⁻¹ N-NO₃⁻; 144.15 g kg⁻¹ total Fe, 3.20 g kg⁻¹ total Mn, 0.108 g kg⁻¹ total Cr, 0.963 g kg⁻¹ total Ni. Mobilised metals by DTPA extraction were: 24.78 mg kg⁻¹ Fe, 107.9 mg kg⁻¹ Mn, 0.40 mg kg⁻¹ Ni and Cr at concentration lower than 0.10 mg kg⁻¹.

The rum vinasse from molasses of sugarcane was collected at the low exit of a distillation column on the 10th December 2002. It was frozen at -20°C. Frozen vinasse was irradiated under γ -gamma source at 15 kGy, and stored at -20°C until experiments began. Dissolved Organic Carbon of the 1:10 diluted vinasse was 1.83 10⁻¹ mol C l⁻¹ and nitrogen concentration of alpha amino acid after HCl (6N) hydrolysis was 1.80 10⁻³ mol N l⁻¹. Vinasse solute content was 4.92 10⁻² mol C l⁻¹ of total carbohydrates, 4.62 10⁻³ mol C l⁻¹ or 1.22 10⁻³ mol N l⁻¹ total proteins, 1.05 10⁻² mol C l⁻¹ total phenols and other soluble organic matter including polyols, small organic acids, phenols and alcohols. The composition in main small carbohydrates, polyols, amino acids, organic acids and alcohols measured by Ionic Chromatography (IC) are presented in the [Table 1](#). Lipids were not found in vinasse. The diluted vinasse had a pH equal to 4.98.

Table 1 : Carbohydrates, polyols, amino acids, organic acids and alcohols measured in 1:10 diluted vinasse (IC measurements).

| Carbohydrates, Polyols (10^{-6} mol l $^{-1}$) | Amino Acids (10^{-12} mol l $^{-1}$) | Organic Acids (10^{-6} mol l $^{-1}$) | Alcohols (10^{-3} mol l $^{-1}$) | | | | |
|---|---|--|---|----------------|------------|-------------|--------|
| Arabinose | 35 ± 4 | Asparagine | 5.7 ± 0.28 | Acetate | 3900 ± 44 | Ethanol | 0.0 |
| Arabitol | 64 ± 1 | Aspratic Acid | 2800 ± 15 | Butyrate | 0.0 | Isopropanol | 19 ± 2 |
| Fructose | 255 ± 9 | Cysteine | 7.2 ± 0.31 | Citrate | 152 ± 21 | | |
| Galactose | 0.0 | Glutamic Acid | 4000 ± 21 | Formate | 1168 ± 84 | | |
| Glucose | 171 ± 11 | Histidine | 14 ± 0.11 | Isoaconitate | 283 ± 23 | | |
| Glycerol | 4100 ± 100 | Isoleucine | 0.2 ± 0.36 | Lactate | 4923 ± 235 | | |
| Inositol | 96 ± 2 | Leucine | 9.5 ± 0.52 | Malate | 212 ± 13 | | |
| Lactose | 0.0 | Lysine | 7.6 ± 1.5 | Oxalate | 124 ± 5 | | |
| Mannitol | 70 ± 25 | Methionine | 5.1 ± 0.03 | Propionate | 83 ± 8 | | |
| Mannose | 0 | Phenylalanine | 18 ± 0.46 | Pyruvate | 119 ± 14 | | |
| Saccharose | 117 ± 5 | Proline | 15000 ± 14 | Tartrate | 616 ± 29 | | |
| Sorbitol | 16 ± 1 | Serine | 0.04 ± 0.01 | Transaconitate | 869 ± 39 | | |
| Ribose | 0.0 | Threonine | 0.0 | | | | |
| Trehalose | 171 ± 3 | Tryptophan | 0.0 | | | | |
| Xylose | 0.0 | Tyrosine | 2.3 ± 0.36 | | | | |

III.2.2 Treatments and measurements

III.2.2.1 Treatment preparation

In order to assess the disappearing rate of carbohydrates, polyols, proteins, small organic acids and alcohols in soil solution, and the relative contributions of retention and biotransformations, 3 treatments were defined:

- treatment (C): 40 g of air dried soil + 67 ml of milliQ water;
- treatment (+S+W): 40 g of air dried soil + 7 ml of 10 g l⁻¹ thimerosal solution + 60 ml of diluted vinasse, subsequently sterilised at thimerosal concentration of 0.25 g l⁻¹;
- treatment (+W): 40 g of air dried soil + 7 ml of milliQ water + 60 ml of diluted vinasse.

Batch incubations were performed in sealed 150 ml flasks. The soil was prehumidified 7 d before the beginning of the incubations, either with 7 ml of milliQ water, or with a 10 g l⁻¹ thimerosal solution for treatment (+S+W). For this treatment, solid thimerosal was dissolved in the 1:10 diluted vinasse (0.25 g l⁻¹), 4 d before vinasse supply. Preliminary tests were done to check the sterility of this treatment. To avoid microbial contamination in this treatment, the addition of the vinasse in the flasks was performed within a laminar airflow hood. All the slurries were placed in anaerobic conditions, by alternating 3 successive cycles of 10 min vacuum and 10 min N₂ replacement. The last N₂ filling of the flask was considered as the initial time of the incubation (t_0). After a final equilibrium at atmospheric pressure, 1 ml of N₂ was replaced with Kr in order to calculate variations in total gas pressures. The flasks were then incubated at 25°C in darkness and continually shaken (150 rpm) (Cooled Orbital Incubator, Gallenkamp, U.K.).

During the incubations, measurements were performed on triplicate flasks (Table 2). They dealt with:

- concentrations in total dissolved organic carbon, carbohydrates, proteins and phenols;
- indicators of microbial activities including gas partial pressures (O₂, CO₂, N₂, H₂, CH₄, Kr), concentrations of identified organic compounds in solution (carbohydrates, polyols, small organic acids, amino-acids and alcohols), and mineral anions and cations (SO₄²⁻, NO₃⁻, NO₂⁻, extractable NH₄⁺, Ca²⁺, Mg²⁺, K⁺, Na⁺);
- total metal (Fe, Mn, Cr and Ni) concentrations in solution, and FeII concentration;
- pH and E_H .

Table 2: Planning of measurements for each treatment.

| Treatment | Dates of measurements | Set 1 (triplicate flasks) Small carbohydrates, Organic Acids, Polyols, Free Amino Acids, D.O.C. SO ₄ ²⁻ , PO ₄ ³⁻ , NO ₃ ⁻ , NO ₂ ⁻ , NH ₄ ⁺ , Fe _{tot} , Mn _{tot} , Cr _{tot} , Ni _{tot} , FeII Gases | Set 2 (triplicate flasks) E _H pH Total NH ₄ ⁺ | Set 3 (triplicate flasks) Total Carbohydrates Total Proteins Total Phenols |
|-----------|-------------------------------|--|---|---|
| (C) | 0.5 h, 3, 7, 14, 21, 30, 40 d | • | • | • |
| (+W) | 0.5 h, 2, 7, 14, 21, 30, 40 d | • | • | • |
| | 1, 4, 10, 12, 14 d | • | • | |
| (+W+S) | 0.5, 3 and 7 h, 1 and 2 d | • (*) | • | • |

III.2.2.2 Analytical procedures

At measurement dates, 2 or 3 sets of 3 flasks were used in each treatment as in [Table 2](#). Measurements were performed on either the gas phase (set 1), the supernatant before sampling (set 2), or the solution after sampling and treatment (sets 1, 2 and 3).

Gas measurements (O_2 , CO_2 , N_2 , N_2O , H_2 , CH_4 and Kr) were performed on a TCD gas chromatograph (CP 3800, Varian, USA) fitted with Porapak Q (80-100 μm mesh, 1.8 m) and sieve molecular (1-5 \AA , 1.8 m) columns coupled to an integration software, Star WS. Carrier gas was He. Relative uncertainties were about 1% (3% for H_2). Before gas sampling, the flasks were vigorously shaken to ensure equilibrium between gas and liquid phases. 0.20 ml of gas was then sampled with a syringe and analysed by TCD chromatography.

To prevent oxidation of the media and pH variation due to CO_2 volatilisation, the pH and the redox potential E_H were measured as soon as the flasks of set 2 were opened under N_2 atmosphere in a glove bag using pH and E_H electrodes (933327 and 90437, respectively, Bioblock Scientific, France). The measurements were performed on the supernatant.

A KCl extraction of NH_4^+ was then performed with soil to KCl solution (1 M) weight ratio equivalent to 1:2.5.

For all flasks, the supernatant sampling was performed under strict N_2 atmosphere, as well as the centrifugation that was operated during 15 min at 1800 g at 4°C (Heraeus, Biofuse stratus, Germany), the filtration at 0.22 μm (Millex GP, Millipore, France), and the distribution of the solution in sterile tubes. The measurement of FeII concentration was immediately done. Tubes intended for the measurement of organic compounds were supplied with NaN_3 to obtain 30 $mg\ l^{-1}$ concentration. Tubes intended to measure total metal concentrations were supplied with 25 μl concentrated HCl in order to avoid oxy-hydroxide precipitation. No chemical was added to the tubes intended for $N-NO_3^-$, $N-NO_2^-$ and $N-NH_4^+$ analyses. All the tubes were then stored at 4°C until measurements.

Colorimetric method (Jasco V-530, UV-VIS Spectrophotometer, Tokyo, Japan) were used for the determination of total carbohydrates at 627 nm ([Dubois et al., 1956](#)), total protein at 595 nm ([Bradford, 1976](#)), and total phenols at 740 nm ([Anderson and Ingram, 1989](#)). The Dissolved Organic Carbon (DOC) was determined using a TOC-meter (TOC 5050A, TC Furnace, Shimadzu, Japan) with the furnace at 680°C.

The concentrations in small organic compounds were determined by IC (Dionex DX 600, U.S.A.) fitted with (i) both CARBOPAQ-PA1 and CARBOPAQ-MA1 columns with

a detector of pulsed amperometry for sugars and polyols, (ii) a CARBOPAQ-MA1 column and a detector of pulsed amperometry for alcohols, (iii) an AMINOPAQ-PA10 column and a detector of pulsed amperometry for amino-acids, and (iv) an IONPAQ-AS11 column and a conductimetric detector for organic acids and mineral anions, such as NO_3^- , SO_4^{2-} , Cl^- and PO_4^{3-} . However, we have retained measurements of NO_3^- , NO_2^- and NH_4^+ performed by spectroscopy (Alliance, EvolutionII, France).

Total concentrations of Fe, Mn, Cr and Ni in solution were determined by ICP-AES (Varian, VISTA-Pro, CCDsimultaneous, France), whereas DPKBH was used as a reagent to determine FeII concentration by spectrophotometry at 660 nm (Bourri  et al., 1999).

III.2.3 Data treatments

III.2.3.1 Representativeness of identified organic compounds

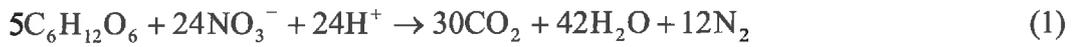
Preliminary treatments aimed to assess (i) the relative contributions of carbohydrates, proteins, phenols, as well as identified organic acids, polyols and alcohols to total soluble organic compounds, and (ii) the relative contributions of identified sugars to the carbohydrate pool, as well as free amino acids to the protein pool. All quantities were expressed in mol C, and also mol N for the protein pool. For conversion of spectroscopic data, the standard molecule of carbohydrate was glucose with 40% C weight content, whereas the standard for protein determination was the Bovin Serum Albumin with about 51% C weight content (Chevallier et al., 2003) and 15.7% N weight content (Sigma Aldrich supplier value). Similarly, the standard of total phenol was tannic acid with 53.7% C weight content.

III.2.3.2 Adsorption/desorption of the organic matter

The treatment (+S+W) permitted to assess adsorption/desorption, including time to reach equilibrium and the proportion of adsorbed solutes. Deviations of treatments (+W) and (+S+W) enabled to evaluate the contribution of microbial absorption/excretion and/or artefacts due to the sterilisation procedure in the treatment (+S+W).

In order to identify the main catabolic pathways and electron donors involved in organic matter degradation and metal reductions, respectively, we have used a few chemical equations to describe actual or equivalent transformations for denitrification, fermentation, metal reduction, formate lysis, SO_4^{2-} reduction, acetogenesis and methanogenesis, based on the review of [Dassonville and Renault \(2002\)](#). We disregarded phenol degradation, since it is difficult to degrade them anaerobically ([Melo et al., 2005](#)). Isopropanol was not considered due to its degradation into acetone ([Fayolle et al., 2003](#)).

Considering the low initial NO_3^- amount, we estimated only maximum consumption of carbohydrates (assimilated to hexoses, expressed in mol C) or acetate ΔQ_{M-D} (mol C) by denitrification, assuming the balance equations:



Thus, we obtain:

$$\Delta Q_{M-D}(\text{C}_6\text{H}_{12}\text{O}_6) = 6 \times \left(\frac{5}{24} \times Q_i(\text{NO}_3^-) \right) \quad (3a)$$

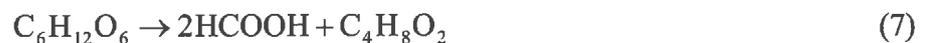
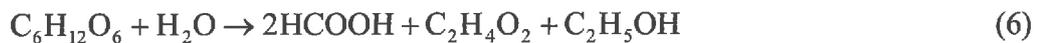
$$\Delta Q_{M-D}(\text{C}_2\text{H}_4\text{O}_2) = 2 \times \left(\frac{5}{8} \times Q_i(\text{NO}_3^-) \right) \quad (3b)$$

where $Q_i(\text{NO}_3^-)$ is the initial amount of NO_3^- (mol). Since the lysis of formate produces H_2 (Eq. (4a)), whereas formate is oxidised by NO_3^- (Eq. (4b)); we have estimated the maximum deficiency in H_2 production due to NO_3^- , ΔQ_{M-D} (mol H_2), (Eq. (5)):

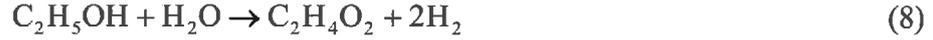


$$\Delta Q_{M-D}(\text{H}_2) = Q_i(\text{NO}_3^-) \quad (5)$$

Based on experimental results, we have considered acetic and butyric fermentation pathways (Eq. (6) and (7)); in contrast, propionic and alcohol pathways have been neglected:



In addition to the lysis of formate described in Eq. (4a), we have accounted for true acetogenic transformations of ethanol and butyrate for H_2 production:



As long as acetate comes only from fermentations and true acetogenesis and is not consumed by acetoclastic methanogenesis or SO_4^{2-} reduction, we may use an I_f index (mol C) corresponding to the equivalent amount of hexose C required to produce butyrate, acetate and ethanol as described in equations (6) to (9):

$$I_f = 6 \times \left\{ (\Delta Q_b/4) + \frac{((\Delta Q_a/2) + (\Delta Q_e/2))}{2} \right\} \quad (10)$$

where ΔQ_b , ΔQ_a and ΔQ_e are the amount variations (mol C) of butyrate, acetate and ethanol, respectively, for a given period. This index was decreased to account for acetate production from anaerobic oxidation of other organic acids, assuming no by-products (e.g. CO_2):

$$I_{\text{rf}} = I_f - \left(\frac{3}{2} \times \Delta Q_{\text{OA}} \right) \quad (11)$$

where I_{rf} is expressed in mol C, ΔQ_{OA} is the amount variation of organic acids (mol C), others than butyrate and acetate.

In order to assess the contribution of fermentations to the initial mineralisation of organic C, we have defined an I_{fm} index (mol C) representing the amount of hexose C required to produce CO_2 by acetic and butyric fermentations with formate lysis or oxidation (Eq. (4), (6) and (7)):

$$I_{\text{fm}} = 3 \times \Delta Q_{\text{CO}_2} \quad (12)$$

where ΔQ_{CO_2} is the deviation in the amount of CO_2 (mol), for a given time interval. Since, the soil did not initially contain carbonates, the total amount of CO_2 in air and solution resulted from microbial production; at a given time, it was estimated through the equation:

$$Q_{\text{CO}_2} = \left(\frac{P(\text{CO}_2)}{R \times T} \right) \times \left(V_a + (V_w \times \alpha_{\text{CO}_2} \times (1 + 10^{\text{pH} - \text{pKa}_1})) \right) \quad (13)$$

where $P(\text{CO}_2)$ is the partial pressure of CO_2 (Pa), R the gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), T the temperature (K), V_a and V_w the volume of air and water in the flask (m^3), α_{CO_2} the solubility of CO_2 in water ($\text{mol m}^{-3} \text{ water} / \text{mol m}^{-3} \text{ air}$), and pKa_1 the first dissociation constant of H_2CO_3 .

Additional indexes I_{FC} , $I_{\text{FC+P}}$ and $I_{\text{FC+P+P}}$ (mol C) were defined for 'carbohydrates', 'carbohydrates+polyols' and 'carbohydrates+polyols+proteins', respectively, in order to assess the contribution of organic pools to fermentations, assuming the same stoichiometry between the initial substrate C and the products as for carbohydrates:

$$I_{fC} = \Delta Q_C \quad (14)$$

$$I_{fC+p} = \Delta Q_C + \Delta Q_P \quad (15)$$

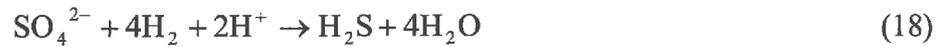
$$I_{fC+p+P} = \Delta Q_C + \Delta Q_P + \Delta Q_P \quad (16)$$

where ΔQ are variations in substrate amounts (mol C) for a given time, and C, p, P are the subscripts for total carbohydrates, polyols and proteins, respectively.

We indirectly assess the maximum gross H_2 production Q_{H_2} (mol H_2) through fermentation (i.e. due to formate lysis) and true acetogenesis (Eq.(4a), (8) et (9)):

$$Q_{H_2} = \frac{2}{3} \times I_{fr} + Q_i(\text{formate}) \quad (17)$$

where $Q_i(\text{formate})$ is the initial quantity of formate (mol C). As seen above, this production may be lowered due to formate oxidation with NO_3^- reduction. Furthermore, SO_4^{2-} reduction can either lower H_2 production or consume H_2 . Since the net H_2 production would be the same whatever the actual pathway as long as actual SO_4^{2-} reduction either use H_2 or produced acetate, we have considered the following equation:



It leads to the $\Delta Q_{M-S}(H_2)$ estimate (mol H_2) of the maximum H_2 consumption through SO_4^{2-} reduction:

$$\Delta Q_{M-S}(H_2) = 4 \times Q_i(SO_4^{2-}) \quad (19)$$

The minimum net production of H_2 , Q_{m-H_2} (mol H_2) can then be evaluated as:

$$Q_{m-H_2} = Q_{H_2} - \{\Delta Q_{M-D}(H_2) + \Delta Q_{M-S}(H_2)\} \quad (20)$$

This quantity was available for metal reduction, homoacetogenesis and hydrogenoclastic methanogenesis. Neglecting the last 2 processes, either $2 \times Q_{m-H_2}$ mol of FeIII could be reduced into FeII, or Q_{m-H_2} mol of MnIV into MnII. Since the 2 metal elements are reduced concomitantly, Q_{m-H_2} was compared with the I_{S-M} index (mol H_2) for the mobilisation of metals:

$$I_{S-M} = \frac{Q_S(\text{FeII})}{2} + Q_S(\text{MnII}) \quad (21)$$

The I_{S-M} -to- Q_{m-H_2} ratio was an estimate of the proportion of reduced metals that are really mobilised, as long as Q_{m-H_2} is a good estimate of H_2 really available for metal reduction.

Before methanogenesis began, indexes I_f , I_{fr} , I_{fC} , I_{fC+p} , I_{fC+p+P} and I_{S-M} , as well as stock variations ΔQ_b , ΔQ_a , ΔQ_e , ΔQ_{OA} , ΔQ_C , ΔQ_P , ΔQ_P and ΔQ_{M-D} were calculated considering

either concentrations in solution between 0.5 h and 2, 7 or 21 d (method 1), or initial values based on vinasse supply and values corrected for retention at previous dates (method 2).

$$Q_i(\text{total}) = \frac{Q_i(\text{solution})}{1 - k_i} \quad (22)$$

where $Q_i(\text{total})$ and $Q_i(\text{solution})$ are the total and solute amounts of compound i (mol C), and k_i is the sorbed - to - solute amount ratio of compound i . To improve these calculations, we should consider i) anabolic consumption, by adding 15% of solute consumption and ii) decrease of CO_2 due to carbonate precipitates, to adjust I_{fm} index.

Since methanogenesis started late after the end of fermentation and acetate production with no detectable H_2 , we compared CH_4 and CO_2 productions to acetate consumption between 21 and 30 days of incubation to check whether H_2 was not significantly used for CH_4 production.

At last, N-balances were also performed to evaluate the net increase in microbial biomass over all the incubation period and check whether N concentration could have limited microbial growth and induce non-symbiotic N fixation. In addition, the fate of exchangeable NH_4^+ enabled to assess the gross microbial death during the end of incubation.

III.2.3.4 Metal speciation

Data treatments aimed to assess (i) the capacity of vinasse compounds to abiotically mobilise metals, (ii) their relative contribution to metal complexation and mobilisation and finally (iii) possible precipitations of reduced metals based on solubility product considerations. Chemical speciations in solution were indirectly assessed using GEOCHEM-PC (Parker et al., 1995). This geochemical model enables to account for equilibria in solution of simple 'metal-ligand' complexes, and the precipitation/dissolution of solids. The database of equilibrium constants combined the database of Parker et al. (1995) and additional constants issued from the NIST standard reference database 46 version 7.0 for the following ligands: propionate, lactate, butyrate, pyruvate, cisaconitate, transaconitate, tryptophan and asparagine.

III.3 Results

III.3.1 Treatment (C)

At 0.5 h of incubation, dissolved organic carbon (DOC) represented less than 1.3% of that in treatment (+W). At the same time, total carbohydrates, polyols, organic acids and phenols represented 22%, 0.5%, 81% and 5%, respectively, of the DOC. Proteins, lipids and alcohols were never detected. Along the 40 d of incubation, the DOC concentration slightly increased from $6.24 \cdot 10^{-4}$ to $1.40 \cdot 10^{-3} \text{ mol C l}^{-1}$. However, this trend was the average of contrasted ones. More precisely, total carbohydrates decreased with time from $1.36 \cdot 10^{-4}$ to $1.10 \cdot 10^{-4} \text{ mol C l}^{-1}$ at 40 d. Identified small carbohydrates and polyols disappeared at 7 d. Organic acid concentrations slightly increased until 21 d of incubation and total phenol concentrations increased from $3.31 \cdot 10^{-5}$ to $6.70 \cdot 10^{-5} \text{ mol C l}^{-1}$ through the incubation.

The CO_2 partial pressure increased linearly from $9.4 \cdot 10^{-4}$ to $1.3 \cdot 10^{-1} \text{ atm}$ at 40 d of incubation; at 30 d, P_{CO_2} corresponded to 21% of its value in treatment (+W). A small peak of N_2O ($1.9 \cdot 10^{-4} \text{ atm}$) appeared at 2 d. No production of CH_4 and H_2 were observed. NO_3^- in solution decreased from $3.8 \cdot 10^{-5} \text{ mol l}^{-1}$ at 0.5 h of incubation to $1.6 \cdot 10^{-6} \text{ mol l}^{-1}$ at 21 d of incubation and then disappeared. Along the first 14 days, SO_4^{2-} concentration increased from $4.8 \cdot 10^{-5} \text{ mol l}^{-1}$ to $8.5 \cdot 10^{-5} \text{ mol l}^{-1}$ at 14 d, and then stabilised around $4.1 \cdot 10^{-5} \text{ mol l}^{-1}$ for the end of the incubation. NH_4^+ concentration increased from $3.8 \cdot 10^{-5} \text{ mol l}^{-1}$ at 0.5 h to $1.3 \cdot 10^{-4} \text{ mol l}^{-1}$ at 40 d of incubation.

The $p\text{H}$ slowly increased from 5.48 to 6.45, whereas E_{H} regularly decreased from 569.4 to 170.4 mV (Fig. 1). Low metal mobilisation was observed compared with treatment (+W): Fe and Mn mobilisations increased through the incubation, reaching $2.3 \cdot 10^{-4}$ and $3.6 \cdot 10^{-4} \text{ mol l}^{-1}$, respectively, at the end. In contrast, Cr and Ni were mobilised immediately, their concentrations fluctuating thereafter around $1.5 \cdot 10^{-7}$ and $4.0 \cdot 10^{-7} \text{ mol l}^{-1}$, respectively, corresponding to 100 and 34.8% of Cr, and 17.8 and 24.6% of Ni mobilised in treatments (+S+W) and (+W), respectively. At 40 d of incubation, Fe, Mn and Ni were mainly in free forms (Fig. 2), whereas nearly all Cr was complexed with CO_3^{2-} .

Figure 1: Evolutions of pH and E_H for treatments (C), (+S+W), and (+W):
a/ pH ; b/ E_H .

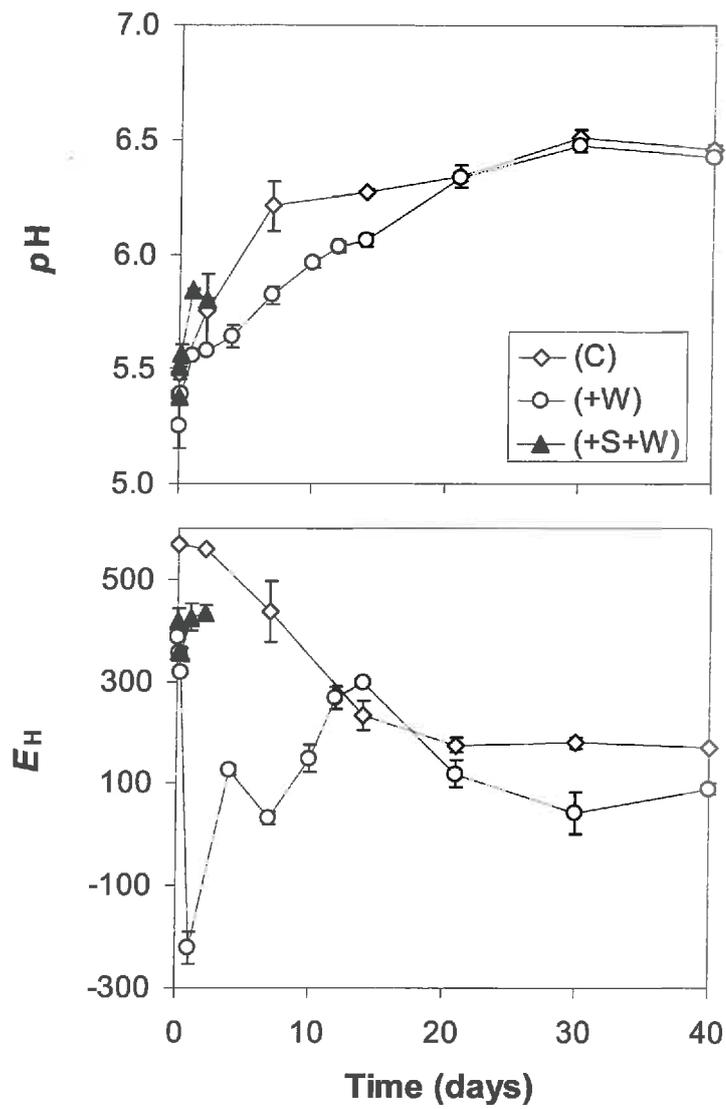
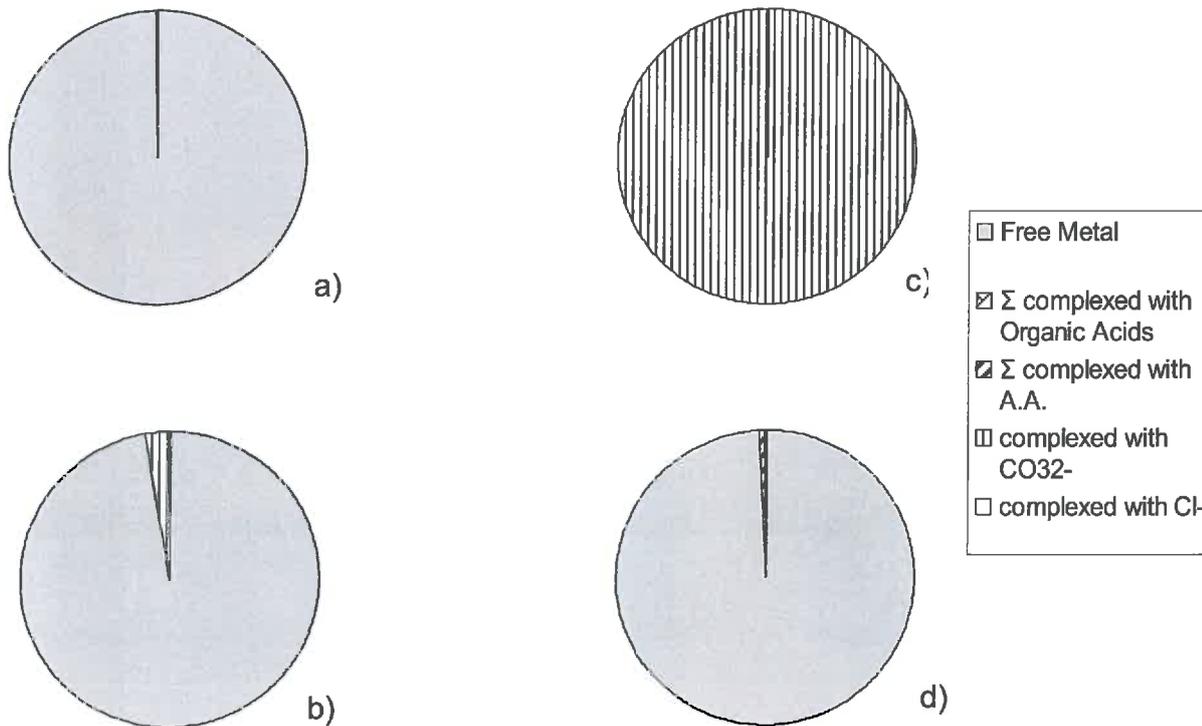


Figure 2: Calculated metal speciation at 40 d of incubation in treatment (C):
a/ Fe, b/ Mn, c/ Cr, d/ Ni.



III.3.2 Treatment (+S+W)

At 0.5 h of incubation, DOC represented 97.6% of that supplied with the vinasse. At this time, total carbohydrates in solution ($3.62 \cdot 10^{-2} \text{ mol C l}^{-1}$) corresponded to 74% of carbohydrates supplied with vinasse (Table 3); it decreased thereafter until $3.17 \cdot 10^{-2} \text{ mol C l}^{-1}$. Different trends along the identified small carbohydrates were observed. Saccharose and trehalose in solution corresponded to 67 and 79% of the supply at 0.5 h of incubation, and then decreased. At 0.5 h of incubation, fructose concentration was equal to 73% of the vinasse supply (Table 3) and remained stable thereafter. In contrast, the concentrations of glucose and arabinose at 0.5 h of incubation were 4.1 and 2.6 times higher than calculated t_0 value and increased thereafter. Similarly, galactose, mannose and ribose appeared in solution at 0.5 h of incubation and remained stable along the incubation, except ribose, from which the concentration increased 2.2 times thereafter.

Polyol concentration at 0.5 h of incubation corresponded to 103% of the vinasse supply (Table 3). At this time, sorbitol, arabitol, glycerol, inositol, and mannitol concentrations corresponded to 71, 75, 97, 113 and 124% of vinasse supply, respectively. Along the incubation, polyol concentrations remained stable from 0.5 h to 2 d.

Total protein concentration at 0.5 h of incubation ($7.18 \cdot 10^{-4} \text{ mol C l}^{-1}$) corresponded to 15% of the vinasse supply (Table 3). It then fluctuated around $6.11 \cdot 10^{-4} \text{ mol C l}^{-1}$. At 0.5 h of incubation, several amino acid concentrations (lysine, asparagine, proline, leucine, histidine, phenylalanine and glutamate) corresponded to vinasse input. Others amino acids disappeared either partially (aspartate and cysteine concentrations at 0.5 h of incubation corresponded only to 52 and 37% of the vinasse supply, respectively) or totally (methionine). Finally, other amino acids (serine, isoleucine, tyrosine and threonine) at 0.5 h of incubation were in higher concentration than expected.

Formate concentration at 0.5 h of incubation corresponded to 42.8% of the supply (Table 3). Thereafter, formate concentrations fluctuated. At 0.5 h of incubation, acetate and lactate concentrations corresponded to 68 and 34.5% of the vinasse supply, respectively, and remained stable thereafter. Butyrate appeared ($8.8 \cdot 10^{-5} \text{ mol l}^{-1}$) at 0.5 h of incubation and propionate concentration was greater than expected. At the same date, other organic acid concentrations represented between 9 and 40% of the vinasse supply (Table 3), at the exception of citrate, which completely disappeared.

Ethanol, absent from the vinasse, appeared in solution ($4.53 \cdot 10^{-3} \text{ mol l}^{-1}$) at 0.5 h of incubation and remained stable thereafter. At 0.5 h of incubation, isopropanol concentration corresponded to 77.6% of the vinasse supply. Isopropanol concentration decreased from $15.2 \cdot 10^{-3} \text{ mol l}^{-1}$ to 0.5 to $13.4 \cdot 10^{-3} \text{ mol l}^{-1}$ at 2 d of incubation.

pH increased from 5.38 to 5.80 during the 2 d of incubation (Fig. 1) and CO_2 partial pressure increased from $2.8 \cdot 10^{-3}$ to $6.5 \cdot 10^{-3} \text{ atm}$. CH_4 , H_2 and N_2O were not detected. NO_3^- and SO_4^{2-} concentrations in solution remained stable around $6.5 \cdot 10^{-5}$ and $1.18 \cdot 10^{-3} \text{ mol l}^{-1}$.

Fe and Mn concentrations were $7.94 \cdot 10^{-6}$ and $5.73 \cdot 10^{-4} \text{ mol l}^{-1}$, respectively, after 0.5 h of incubation. Mn concentration increased linearly along the 2 d of incubation, whereas no particular trend was observed for Fe, considering the variability between replicates (Fig. 3). Maximum mobilised Fe and Mn concentrations corresponded to 0.18% and 22%, respectively, of maximum values in treatment (+W). From 0.5 h to 2 d of incubation, Cr and Ni concentrations stabilised around $1.37 \cdot 10^{-7}$ and $9.84 \cdot 10^{-7} \text{ mol l}^{-1}$, respectively (Fig. 3), these values corresponding to 34.8 and 99.3% of maximum Cr and Ni concentrations, respectively, in treatment (+W). Speciation calculations at 0.5 h indicated that 83.5% Fe(II) were as a free

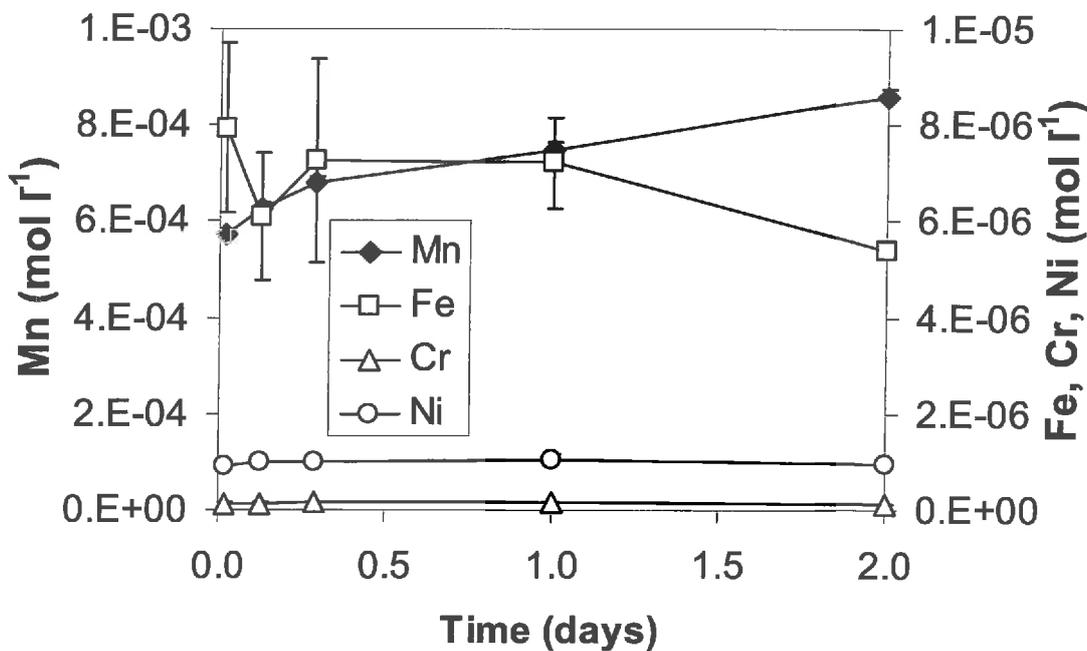
Table 3: Adsorption/desorption of the organic matter.

| Organic Compounds (by pool or individual) | Solution Ratio | | Organic Compounds (by pool or individual) | Solution Ratio | | Organic Compounds (by pool or individual) | Solution Ratio | |
|--|----------------|-------------|--|----------------|-------------|--|----------------|-------------|
| | (+S+W) (%) | (+W) (%) | | (+S+W) (%) | (+W) (%) | | (+S+W) (%) | (+W) (%) |
| COD | 97.6 | 58.2 | Σ <i>Organic Acids</i> | 38.5 | 46.4 | Σ <i>Polyols</i> | 103.6 | 82.8 |
| Total carbohydrates | 79.5 | 74.1 | Acetate | 68.0 | 74.1 | Arabitol | 75.0 | 91.8 |
| Total proteins | 17.4 | 20.8 | Butyrate* | - | - | Glycerol | 97.0 | 72.9 |
| Total phenols | 62.1 | 62.0 | Citrate | 0.0 | 0.0 | Inositol | 112.7 | 99.4 |
| | | | Formate | 42.8 | 63.5 | Mannitol | 124.1 | 108 |
| Σ <i>Identified small sugars</i> | 141.4 | 165.7 | Isoaconitate | 13.0 | 12.1 | Sorbitol | 71.2 | 105.3 |
| Arabinose | 255.0 | 165.3 | Lactate | 34.5 | 41.8 | | | |
| Fructose | 73.1 | 75.7 | Malate | 38.3 | 49.6 | Σ <i>Alcohols</i> | 123.4 | 74.9 |
| Galactose* | - | - | Oxalate | 19.9 | 45.2 | Ethanol* | - | - |
| Glucose | 408.3 | 352.3 | Propionate | 145.0 | 165.4 | Isopropanol | 102.9 | 68.5 |
| Mannose* | - | - | Pyruvate | 26.1 | 30.1 | | | |
| Saccharose | 67.0 | 83.7 | Tartrate | 9.9 | 45.4 | | | |
| Ribose* | - | - | Transaconitate | 25.0 | 21.4 | | | |
| Trehalose | 78.5 | 67.1 | | | | | | |

(* *Compounds detected in the slurry at 0.5 h, but not detected in the added vinasse.*)

in treatment (+W). Speciation calculations at 0.5 h indicated that 83.5% Fe(II) were as a free cation, whereas 9.4, 3.1, 4.0% were complexed with organic acids, amino acids and mineral anions, respectively. Similarly, 85.3% Mn(II) were as a free cation, whereas 7.7, 0.02 and 7.0% of Mn were complexed with organic acids, amino acids and mineral anions, respectively. At the same date, Cr was totally complexed with butyrate, whereas 59.1% Ni were as a free cation and 30, 7.1 and 3.7% Ni were complexed with organic acids, amino acids and mineral anions, respectively.

Figure 3: Evolution over time of mobilised Fe and Mn over the 2 d of incubation in treatment (+S+W).

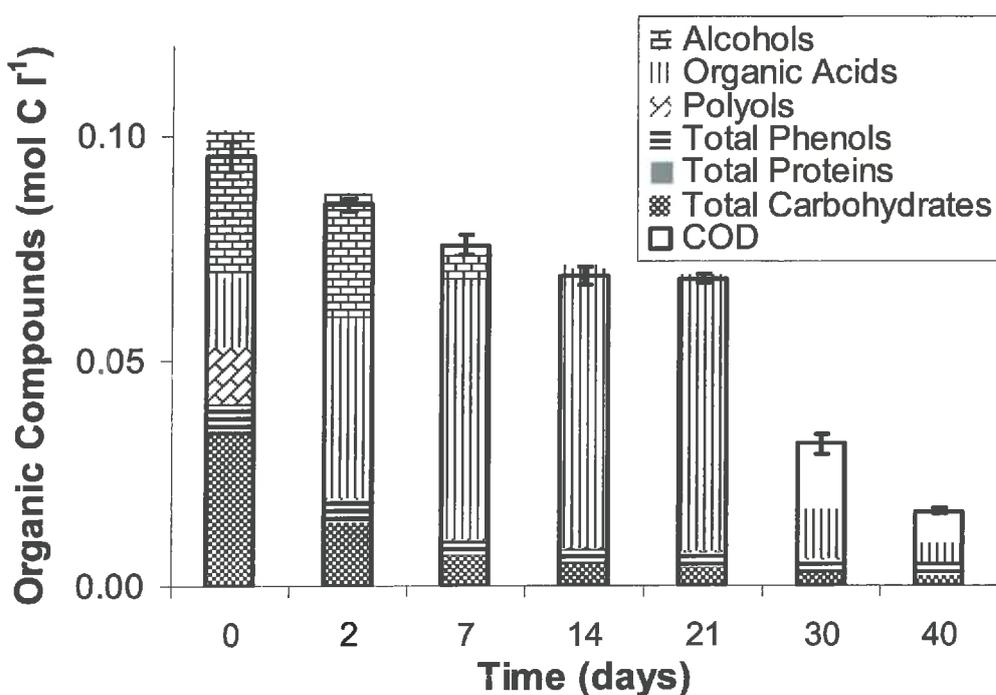


III.3.3 Treatment (+W)

At 0.5 h of incubation, DOC represented 58% of that supplied with the vinasse. At this time, total carbohydrate concentration ($3.37 \cdot 10^{-2} \text{ mol C l}^{-1}$) corresponded to 74% of the vinasse supply (Table 3), whereas the sum of small identified carbohydrate concentrations was higher than the vinasse supply (109.5%). More precisely, saccharose, fructose and trehalose concentrations corresponded to 83.7, 75.7 and 67.0%, respectively, of the t_0 value. In contrast, glucose and arabinose concentrations represented to 352.2 and 165.3%,

respectively, of vinasse supply (Table 3). As in treatment (+S+W), ribose and galactose appeared in excess at 0.5 h of incubation. At the same time, polyol concentration was slightly lower than the calculated t_0 value (82.8%). Identified small carbohydrate and polyol fates were thereafter similar with initial rapid decreases until 2 d, followed by reduced decreases until their disappearance (Fig. 4). At 7 d, polyols were no more present, whereas the decrease of total carbohydrate slowed down, their concentration reaching $2.0 \cdot 10^{-3} \text{ mol C l}^{-1}$ after 40 d of incubation.

Figure 4: Contribution of the identified organic compounds to total soluble organic compounds.



Total protein concentration at 0.5 h of incubation ($8.6 \cdot 10^{-4} \text{ mol C l}^{-1}$) corresponded to 20.8% of the vinasse supply. At this time, leucine was more abundant than expected, whereas several free amino acids were adsorbed on solid phases, following the order with increasing retention order: phenylalanine < glutamate < methionine < aspartate < asparagine < histidine. Others amino acids were not detectable. After the first 0.5 h, protein concentration quickly decreased to become undetectable at 7 d of incubation.

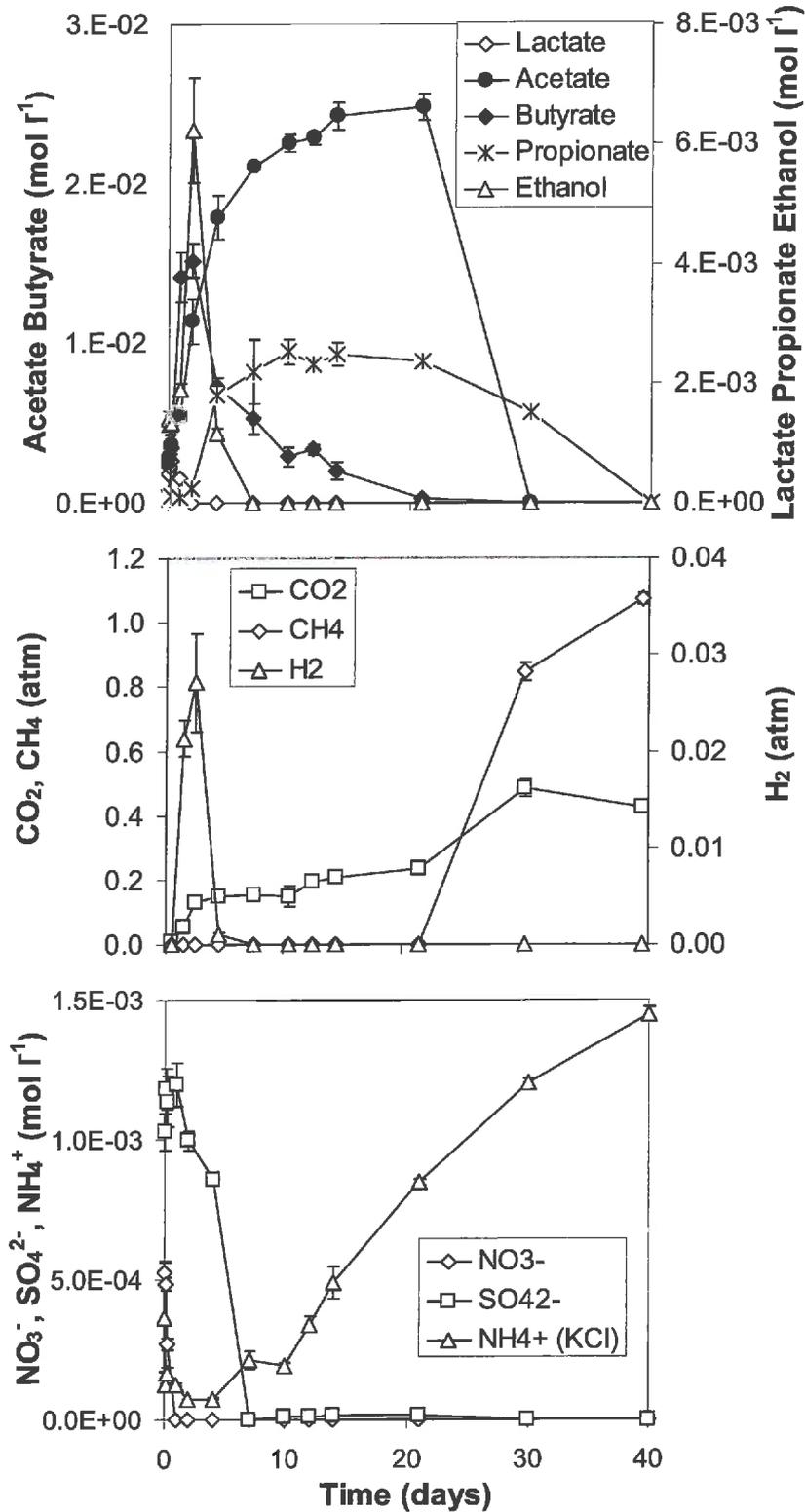
Organic acids in solution at 0.5 h of incubation represented 46.4% of the vinasse input (Table 3). At this time, formate and acetate concentrations represented 64% and 74%, respectively, of the vinasse supply. Formate concentration was ever lower than $7.16 \cdot 10^{-3}$

4 mol l^{-1} and it disappeared at 30 d of incubation (Fig. 5a). Acetate increased from $2.6 \cdot 10^{-3}$ to $2.11 \cdot 10^{-2} \text{ mol l}^{-1}$ from 0.5 h to 7 d of incubation, then stabilised until 21 d, and finally decreased to disappear (Fig. 5a). Lactate concentration at 0.5 h of incubation corresponded to 42% of the vinasse supply and then decreased until disappearance at 2 d of incubation (Fig. 5a). At 0.5 h of incubation, propionate concentration corresponded to 182.2% of the vinasse supply. After a lag phase of 1 d, propionate concentration increased until 10 d of incubation, then stabilised until 21 d and finally disappeared at 40 d (Fig. 5a). Butyrate appeared at 0.5 h of incubation and its concentration increased from $8.8 \cdot 10^{-5}$ to $4.05 \cdot 10^{-3} \text{ mol l}^{-1}$ at 2 d of incubation (Fig. 5a). After this date, butyrate concentration decreased until disappearance at 30 d of incubation. At 0.5 h of incubation, organic acids in solution represented between 12 and 50% of the vinasse supply. All these organic acids decreased versus time until their disappearance at different dates.

Ethanol in solution increased from $1.41 \cdot 10^{-3} \text{ mol l}^{-1}$ (0.5 h) to $6.2 \cdot 10^{-3} \text{ mol l}^{-1}$ (2 d) and then decreased to disappear at 7 d of incubation (Fig. 5a). Isopropanol concentration at 0.5 h of incubation ($1.01 \cdot 10^{-2} \text{ mol l}^{-1}$) corresponded to 51.6% of the vinasse input. After this date, isopropanol concentration increased to reach a maximum value of $1.85 \cdot 10^{-2} \text{ mol l}^{-1}$ at 2 days and then decreased until its disappearance at 14 d of incubation. Total phenol concentration decreased during the first 7 days, from $5.86 \cdot 10^{-3}$ to $2.88 \cdot 10^{-3} \text{ mol C l}^{-1}$ and tended to stabilise thereafter.

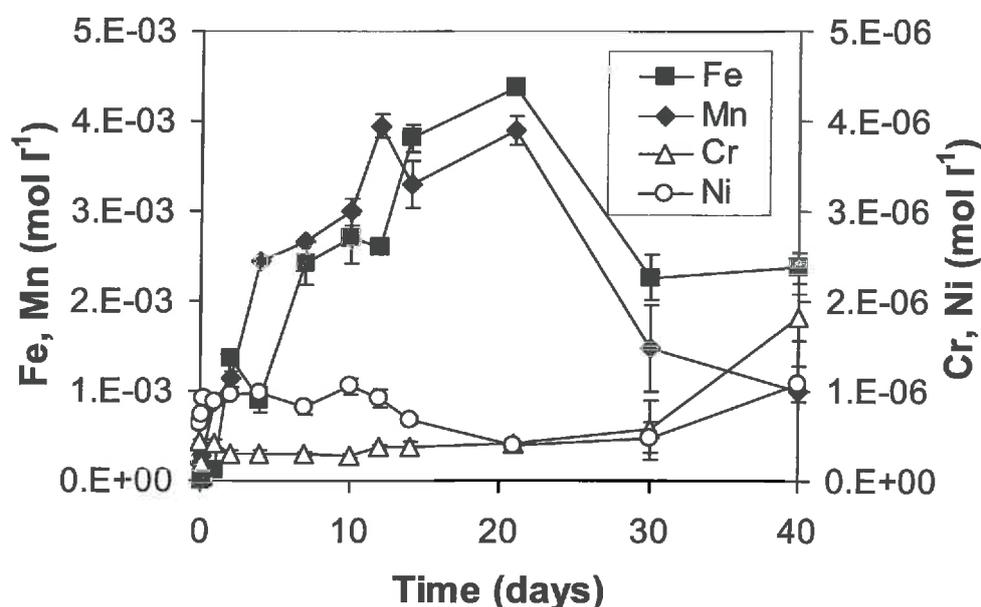
CO_2 partial pressure greatly increased during the first 7 d of incubation (0.16 atm); it thereafter stabilised until 21 d of incubation, and increased again between 21 and 30 d of incubation to reach 0.48 atm at 30 d (Fig. 5b). Finally, a decrease in CO_2 partial pressure was observed between 30 and 40 d (0.42 atm). After 21 d of incubation, an emission of CH_4 was observed (Fig. 5b). At 40 d, CH_4 was equal to 1 atm. An H_2 peak was detected between 7 h and 4 d of incubation, reaching a maximum value of 0.027 atm at 2 d (Fig. 5b). A peak of N_2O ($7.5 \cdot 10^{-5} \text{ atm}$) was observed after 3 h of incubation. Simultaneously, NO_3^- in solution decreased from $5.3 \cdot 10^{-4} \text{ mol l}^{-1}$ at 0.5 h of incubation and disappeared after 1 d (Fig. 5c). At 0.5 h of incubation, SO_4^{2-} concentration was equal to $1.03 \cdot 10^{-3} \text{ mol l}^{-1}$. After 1 d of incubation, SO_4^{2-} concentration decreased to disappear at 7 d (Fig. 5c). NH_4^+ concentration decreased from $3.66 \cdot 10^{-4}$ to $7.4 \cdot 10^{-5} \text{ mol l}^{-1}$ at 4 d of incubation (Fig. 5c). Then, it increased to reach $1.44 \cdot 10^{-3} \text{ mol l}^{-1}$ at 40 d of incubation.

Figure 5: Evolution of indicators of microbial activities in treatment (+W):
 a/ acetate, butyrate, lactate, propionate, ethanol;
 b/ CO₂, CH₄ and H₂;
 c/ NO₃⁻, NH₄⁺ and SO₄²⁻.



At 0.5 h of incubation, Fe and Mn concentrations in solution were $8.6 \cdot 10^{-6}$ and $1.9 \cdot 10^{-4} \text{ mol l}^{-1}$, respectively. Fe and Mn concentrations increased to reach $4.37 \cdot 10^{-3}$ and $3.90 \cdot 10^{-3} \text{ mol l}^{-1}$, respectively, at 21 d of incubation (Fig. 6), and then decreased to $2.37 \cdot 10^{-3} \text{ mol l}^{-1}$ of Fe and $1.01 \cdot 10^{-3} \text{ mol l}^{-1}$ of Mn at 40 d. At 0.5 h of incubation, 85.7% of Fe in solution were as a free cation, whereas 11.0, 0.1 and 3.1% were complexed with organic acids, amino acids and SO_4^{2-} , respectively. At this time, 86.5% of Mn in solution were as a free cation, whereas 7.7 and 5.8% were complexed with various organic acids and mineral anions, respectively. At 21 d of incubation, calculations that were performed without considering precipitation, gave the following speciations (Fig. 7) : 81.0% of Fe were as a free cation and 18.8, 0.1 and 0.1% were complexed with organic acids, amino acids and mineral anions, respectively, and 65.7% of Mn were as a free cation and 16.7 and 17.6% of Mn were complexed with organic acids and with mineral anions, mainly CO_3^- .

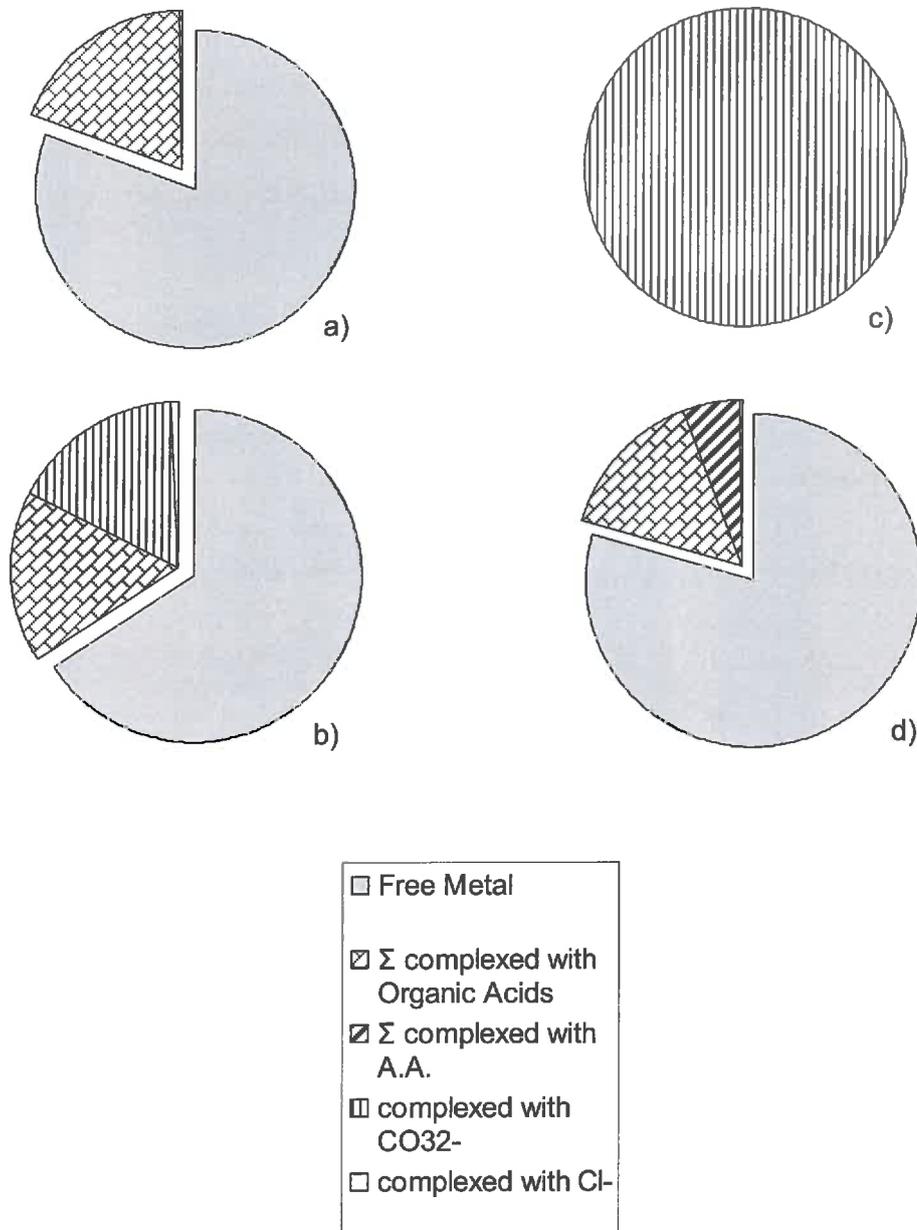
Figure 6: Evolution over time of mobilised Fe, Mn, Cr and Ni over the 40 d of incubation in treatment (+W).



Cr and Ni were mobilised since the beginning of the incubation, and oscillated around $3.4 \cdot 10^{-7}$ and $8.2 \cdot 10^{-7} \text{ mol l}^{-1}$ respectively (Fig. 6). At 0.5 h of incubation, Cr was totally complexed with butyrate, whereas it was complexed with CO_3^{2-} at 21 d (Fig. 7). At 0.5 h of incubation, 59.6% of Ni in solution were as a free cation, whereas 29.4, 8.1 and 2.9% of Ni were complexed with organic acids, amino acids and mineral anions, respectively. At 21 d of

incubation, Ni speciation (Fig. 7) changed with 79.3% as free cation, and 14.8, 5.7 and 0.2% complexed with organic acids, amino acids and anions, respectively.

Figure 7: Calculated metal speciation at 21 d of incubation in treatment (+W):
a/ Fe, b/ Mn, c/ Cr, d/ Ni.



Cr and Ni were mobilised since the beginning of the incubation, and oscillated around $3.4 \cdot 10^{-7}$ and $8.2 \cdot 10^{-7} \text{ mol l}^{-1}$ respectively (Fig. 6). At 0.5 h of incubation, Cr was totally complexed with butyrate, whereas it was complexed with CO_3^{2-} at 21 d (Fig. 7). At 0.5 h of incubation, 59.6% of Ni in solution were as a free cation, whereas 29.4, 8.1 and 2.9% of Ni were complexed with organic acids, amino acids and mineral anions, respectively. At 21 d of incubation, Ni speciation (Fig. 7) changed with 79.3% as free cation, and 14.8, 5.7 and 0.2% complexed with organic acids, amino acids and anions, respectively.

The proportions of reduced Fe and Mn that were mobilised have been indirectly assessed from Fe and Mn evolutions in solution (Fig. 6): assuming that precipitation began at 7 d and reduction stopped at 21 d, we estimated the amount of FeII and MnII precipitated over the 7 - 30 d period from the mean rate of actual precipitation between 21 and 30 d. Since mobilised FeII and MnII amounts did not greatly vary thereafter, we compared these amounts to the sums of these amounts and the corresponding precipitated amounts. We thus estimated that 31% and 13% of reduced FeII and MnII, respectively, remained in solution at the end of the incubation. Maximum gross H_2 production ($Q_{\text{H}_2} = 3.23 \cdot 10^{-3} \text{ mol H}_2$) lowered by maximum consumptions of H_2 by denitrification ($\Delta Q_{\text{M-D}}(\text{H}_2) = 4.71 \cdot 10^{-5} \text{ mol H}_2$) and by SO_4^{2-} reduction ($\Delta Q_{\text{M-S}}(\text{H}_2) = 4.82 \cdot 10^{-4} \text{ mol H}_2$), resulted to minimum available H_2 for metal reduction, $Q_{\text{m-H}_2}$, of $2.70 \cdot 10^{-3} \text{ mol H}_2$. At 21 d of incubation, mobilised Fe and Mn quantities were maximum and equal to $2.93 \cdot 10^{-4}$ and $2.61 \cdot 10^{-4} \text{ mol}$, respectively, and lead to an $I_{\text{S-M}}$ index for the metal mobilisation equal to $4.08 \cdot 10^{-4} \text{ mol H}_2$. The $I_{\text{S-M}}$ index corresponded to 15.1% of $Q_{\text{m-H}_2}$.

Index values depend on calculation method, i.e. with or without accounting for retention on solids (Table 4). Method 1 led to I_f values about 24 - 31% lowered compared with the method 2. I_{rf} value were similar using the 2 methods, since the correction term ΔQ_{OA} offset deviations due to the initial adsorption of supplied compounds. Considering indexes calculated with method 2, $I_{\text{fm}}/I_{\text{rf}}$ was equal to 57.1, 56.0 and 96.2%, over the 3 periods 0 – 2 d, 0 – 7 d and 0 – 21 d respectively. Considering the contribution of organic pools, $I_{\text{fc+p+p}}$ corresponded to 65.3% of I_{rf} by the method 1 and 84.3% by the method 2. Additional calculations (Table 4) on pool contribution showed that degradation of polyols, proteins and carbohydrates occurred in 2, 7 and 21 d, respectively.

Table 4: Indexes at different dates of incubation.

| Indexes | Method 1 | | | Method 2 | | | Method 2 | | |
|-----------------------|--------------------------|-------------|--------------|--------------------------|---------|----------|-------------|-----------|------------|
| | 0.5 h → 2 d | 0.5 h → 7 d | 0.5 h → 21 d | 0 → 2 d | 0 → 7 d | 0 → 21 d | 0.5 h → 2 d | 2 d → 7 d | 7 d → 21 d |
| | (10 ⁻³ mol C) | | | (10 ⁻³ mol C) | | | (%) | | |
| I_f | 4.32 | 3.96 | 4.18 | 5.26 | 5.58 | 6.05 | | | |
| I_{rf} | 3.78 | 4.08 | 4.41 | 3.24 | 4.14 | 4.71 | | | |
| I_{fm} | 1.85 | 2.32 | 4.53 | 1.85 | 2.32 | 4.53 | | | |
| I_{fc} | -1.33 | -1.79 | -1.98 | -1.79 | -2.41 | -2.67 | | | |
| I_{fc+pp} | -2.17 | -2.62 | -2.82 | -2.81 | -3.42 | -3.69 | | | |
| I_{fc+pp} | -2.22 | -2.68 | -2.88 | -3.04 | -3.70 | -3.97 | | | |
| $\Delta Q_C / I_{rf}$ | | | | | | | 55.16 | 69.57 | 43.85 |
| $\Delta Q_p / I_{rf}$ | | | | | | | 31.45 | -1.10 | 1.12 |
| $\Delta Q_P / I_{rf}$ | | | | | | | 7.23 | 4.75 | 0.00 |

III.4 Discussion and conclusions

III.4.1 Representativeness of organic pools and identified compounds in these pools

In treatment (+W), the contributions of carbohydrates, polyols, organic acids, alcohols and phenols to DOC were 35.7, 13.6, 15.5, 34.7 and 6.4%, respectively, at 0.5 h of incubation. Total protein contribution was low (Fig. 4). The first days of incubation were thereafter marked by the decreases of polyols and carbohydrates (Fig. 4), that were concomitant to the production of ethanol and the accumulation of volatile fatty acids. After 21 d of incubation, the decrease in DOC was mainly due to the decrease in acetate, butyrate and propionate concentrations (Fig. 4). Except for dates after 21 d, the sum of identified organic pools exceeded slightly DOC; i.e. 101 – 106%, suggesting that unidentified organic compounds such as humic and fulvic acids did not greatly affect soil behaviour.

Identified small carbohydrates represented 21.7% of total carbohydrate concentration in treatment (+W) after 0.5 h of incubation, and less than 0.5% after 7 d (Fig. 4), suggesting that most of carbohydrates were unidentified oligo and polymers. The hydrolysis of these compounds could thus have been the limiting step in their microbial consumption rate, as it is generally recognised (Glissmann and Conrad, 2002).

Free amino acid contribution to protein pool was unfortunately difficult to estimate, since there were obviously other products that contributed to the IC peaks for at least arginine and alanine.

III.4.2 Adsorption / desorption versus absorption / excretion of organic compounds

With regard to organic C supplied with vinasse, 79.5, 103.6, 38.5, 123.4, 62.1 and 17.4% of carbohydrates, polyols, organic acids, alcohols, phenols and proteins, respectively, were recovered in the solution at 0.5 h of incubation in treatment (+S+W). These values were similar for treatment (+W): 74.1, 82.1, 46.4, 74.9, 62.0 and 20.8% of carbohydrates, polyols, organic acids, alcohols, phenols and proteins, respectively. These similarities suggest abiotic

adsorption/desorption. However, calculations on pools have hidden differences on each compound (Table 3). For example, although total carbohydrates corresponded at 0.5 h to 74 – 80% of the supply value in both treatments, arabinose, glucose, mannose and ribose were in excess at 0.5 h, particularly in treatment (+S+W). Similarly, the adsorption of about 38 - 47% organic acid pool did not reflect individual trends, for propionate, acetate, butyrate, and trans- and iso-aconitate, and pyruvate (Table 3). Moreover, comparison between treatments (+S+W) and (+W) resulted in differences for polyol and alcohol pools, and each compound: e.g. mannose, ribose, glycerol, inositol and mannitol concentrations in treatment (+S+W) were greater than in treatment (+W). Although the entire polyol pool in treatment (+S+W) was larger than in (+W), the opposite was observed for arabitol and sorbitol. Greater concentrations of tartrate, oxalate and formate were observed at 0.5 h in treatment (+W) than (+S+W). Differences between treatments (+S+W) and (+W) suggest that not only abiotic processes contribute to solution characteristics, and microbial transformations have an effect on solid phases adsorption/desorption and possible excretions.

III.4.3 Metabolic pathways

Denitrification and dissimilatory reduction of NO_3^- into NH_4^+ were neglected with regard to organic matter degradation or lack in H_2 production. Indeed, denitrification could have contributed to the consumptions of less than either 1.9% of carbohydrates ($\Delta Q_{\text{M-D}}(\text{C}_6\text{H}_{12}\text{O}_6) = 5.89 \cdot 10^{-5} \text{ mol C}$) supplied with vinasse, or 1.8% of acetate amount ($\Delta Q_{\text{M-D}}(\text{C}_2\text{H}_4\text{O}_2) = 5.89 \cdot 10^{-5} \text{ mol C}$) at 21 d of incubation. Similarly, the initial reduction of NO_3^- into NO_2^- with the concomitant oxidation of formate could have prevented the production of less than 1.5% ($\Delta Q_{\text{M-D}}(\text{H}_2) = 4.71 \cdot 10^{-5} \text{ mol}$) of the calculated H_2 production through formate lysis and true acetogenesis ($Q_{\text{H}_2} = 3.23 \cdot 10^{-3} \text{ mol}$). Although the supply of easily decomposable organic matter favours dissimilatory reduction of NO_3^- into NH_4^+ at the expense of denitrification (Fazzolari et al., 1990, 1998; Tiedje, 1988), accounting for this last process would not have changed the small effect of N oxides on the fate of organic matter and H_2 .

In contrast, acetic and butyric fermentations, as well as associated true acetogenic transformations were greatly involved in the initial fate of organic matter, as it can be seen from the accumulation of butyrate and acetate (Fig. 5a), in agreement with previous similar batch experiments (Dassonville et al., 2004). Whatever the method of index estimation, for the first 21 d of incubation, the I_{fm} index represented about 100% of the I_{rf} index, suggesting

that fermentation and true acetogenic transformation of butyrate and ethanol explained most of the fate of organic matter during this period. On the opposite, propionic fermentations may probably be neglected, since the production of propionate was closely linked with the disappearance of initially supplied lactate that can evolve to propionate and acetone (Pelmont, 1993).

Using method 1, consumptions of the pools “Carbohydrates, polyols and Proteins” (I_{fC+p+p}) did not balance productions by fermentations of acetate, butyrate and ethanol (I_{f}), all the more anabolic consumption would have increased this deviation, by decreasing I_{fC+p+p} . C balance was better assessed by method 2, which considers all organic compounds retained on solids, accounting for retention and excretion. Using this method, I_{f} corresponded to 77.9% of the I_f value due to the contribution of small organic acids to 22.1% of acetate production through anaerobic oxidations. The I_{fm}/I_{f} ratios were equal to 57.1, 56.0 and 96.2% for the 0 - 2 d, 0 - 7 d and 0 - 21 d respectively; we may assume that formate was not instantaneously lysed into CO_2 and H_2 , even if its concentration in solution remained low throughout the incubation. Since formate can be absorbed by microbial cells, all the more microbial population has increased. Indeed, the abiotic adsorption of formate observed in treatment (+S+W) (42 - 63%) (Table 3) could not explain alone its low concentration. Comparison of I_{fC} , I_{fC+p} and I_{fC+p+p} indexes against I_{f} clearly indicated that not only most of carbohydrates, but also most of polyols and proteins participate to the initial accumulation of fermentation products, all the more these catabolic transformations were coupled with anabolic ones that would have increased the consumption of organic-C of about 15%. As revealed by ΔI_{fC} , ΔI_{fp} and ΔI_{fp} determination, the different pools did not contribute initially to the catabolism proportionally to their abundance in the vinasse, according to the increasing order of degradation rate : proteins < carbohydrates < polyols.

Since all carbohydrates, polyols and proteins were required to explain fermentations and acidogenic transformations, the reduction of metals (FeIII, MnIV) was *a priori* not directly coupled to oxidation of organic matter but rather to H_2 oxidation. During the 21 first days of incubation, minimum available H_2 for metal reduction, Q_{m-H_2} , was equal to $2.70 \cdot 10^{-3}$ mol H_2 . During this period, we did not consider that methanogenesis, N_2 non-symbiotic fixation and homoacetogenesis affected H_2 fate. Indeed, CH_4 production was observed only thereafter and closely linked with acetate consumption and CO_2 production from 21 d to 30 d; methanogenesis as well as the acetogenic consumption of propionate beyond 21 d indicated that H_2 pressure was very low. Secondly, the lowest NH_4^+ concentration ($1 \cdot 10^{-4}$ mol $N l^{-1}$) observed along the incubation did not reach limiting conditions of N availability (Shi et

Norton, 2000, in case of nitrification). Finally, we did also not invoke homoacetogenesis, within sight of acetate concentration and CO₂ partial pressure evolution that never decreased before 21 d of incubation and $I_{\text{fm}}/I_{\text{rf}}$ ratio was nearly equal to 1 at 21 d using the method 2, even if the occurrence of this process can not be totally excluded. The $I_{\text{S-M-to-Q}_{\text{m-H}_2}}$ ratio can be viewed as an indicator of the proportion of reduced metal that was mobilised. This ratio equalled 15.1%. Other studies already noticed that only a few percent of reduced FeII was in solution (Dassonville, 2003; Ona-Ngema, 2003). This result agrees with geochemical calculations, using GEOCHEM-PC) that indicates that FeII could precipitate as siderite (FeCO₃) after 7 d of incubation. According to calculations on Figure 6, we roughly estimated that 31% and 13% of reduced FeII and MnII, respectively, remained in solution at the end of the incubation.

III.4.4 N balance

Total N in solution of treatment (+W) (N-NO₃⁻ + N-TotalNH₄⁺ + N-proteins) were 2.0 10⁻³ and 1.45 10⁻³ mol N l⁻¹ at 0.5 h and 40 d of incubation, respectively. Thus, 0.54 10⁻³ mol N l⁻¹ would have been immobilised, neglecting denitrification with regard to dissimilatory reduction of NO₃⁻ into NH₄⁺ (Fazzolari et al., 1990, 1998). Considering that anabolic activities consumed 15% of the added organic carbon and N represent 10.4% of total biomass, biomass gross increase would then have been of 0.287 g l⁻¹, including 2.98 10⁻² g N l⁻¹ (i.e. 2.13 10⁻³ mol N l⁻¹). This value approximately equalled the initial available N. Moreover, since most of microbial activities occurred during the first 10 days of incubation, it explained the low total NH₄⁺ content of the solution during this period (lowest concentration 8.0 10⁻⁵ mol l⁻¹). From 10 to 40 d of incubation, NH₄⁺ production (1.26 10⁻³ mol N l⁻¹) should correspond to the death and mineralisation of about 67% of the created biomass. Neglecting the initial soil biomass and considering these deaths over a period of 30 d, it would correspond to a maintenance coefficient of about 4.3 10⁻⁷ s⁻¹, that is low with regard to published values, although in the same range (Dassonville et al., 2004). The deviation between the gross immobilised N and its subsequent partial mineralisation would then equal 6.3 10⁻⁴ mol N l⁻¹ that is close to the 5.4 10⁻⁴ mol N l⁻¹ estimated from the N balance between the beginning and the end of the incubation. This deviation could partly results from initial soil microbial biomass that was not included in N balance calculation.

III.4.5 Metal speciation

Fe and Mn concentrations increased from the beginning to 21 d of incubation, and decreased thereafter until 30 d to stabilise at the end of incubation; speciation calculations have indicated that mobilised FeII and MnII were mainly as free cations, 81.0 and 65.7%, respectively (Cambier and Charlatchka, 1999). Therefore, the disappearance of acetate after 21 d can not explain the mobilisation of these metals. More probably, the increase, in their mobilisation and in the CO₂ partial pressure through the incubation, enabled thermodynamically the precipitation of siderite (FeCO₃) and rhodocrosite (MnCO₃) after a date between 7 and 10 d of incubation. Before 21 d, we assume that precipitations coexisted with mobilisation, even if reduction of FeIII and MnIV led to a net increase of mobilised FeII and MnII. After 21 d, the reduction of FeIII and MnIV probably stopped due to the very low pressure of H₂, that was confirmed by CH₄ production and acetogenesis of propionate and butyrate. Cr and Ni were mobilised since the beginning of incubation in treatment (+W), as in treatment (+S+W), their concentration in solution fluctuating thereafter with non large variations (Fig. 3, 6). The contribution of Cr reduction to anaerobic microbial activities could be neglected within sight of its concentration in solution compared to those of Fe and Mn. Ni was mobilised with no reduction. Speciation calculations at 0.5 h of incubation indicated a complete complexation of Cr with butyrate supplied with vinasse, then CO₃²⁻ progressively substituted to butyrate as ligand, simultaneously to its degradation. Ni speciation for treatments (+S+W) and (+W) were nearly identical with about 59% of Ni as a free cation and 30%, 8% and 3% complexed with organic acids, amino acids and anions, respectively. Along the incubation, the consumption of the organic acids and amino acids was correlated to the increase of Ni proportion, as a free cation.

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IV Contributions des réducteurs du FeIII à la réduction du FeIII

Dans le travail résumé dans le chapitre précédent, la réalisation de bilans de matière a montré que la réduction des métaux se faisait principalement avec l'oxydation concomitante de H₂ ou de précurseurs de H₂ (formiate, propionate, butyrate et éthanol), l'oxydation de ces derniers s'arrêtant alors au stade acétate comme au cours de l'acétogénèse réductrice de protons. Toutefois, la réalisation de ces bilans ne nous permet pas de trancher sur les acteurs et les processus sous-jacents à ces réductions (microbiennes avec gain d'énergie par des réducteurs de FeIII qui peuvent alors satisfaire leur besoin en énergie par ces réductions, microbienne sans rendement énergétique par des bactéries comme les fermentaires, ou abiotique suite à la production microbienne d'autres composés réduits comme les sulfures). En effet, si la réduction des métaux a été longtemps considérée comme abiotique, à la fin des années 70, il a été montré qu'elle s'effectuait préférentiellement par voie microbienne. Si de nombreuses recherches ont porté sur l'étude des acteurs des réductions métalliques et des processus impliqués dans ces réductions, des interrogations restent en suspens (i) dans la compréhension des interactions entre réductions du FeIII et du MnIV et dynamique des réducteurs du FeIII, (ii) sur les contributions relatives des réactions microbiennes et abiotiques à la réduction du FeIII et (iii) sur l'impact de la présence de donneurs d'électrons et d'accepteurs d'électrons autres que FeIII et MnIV. Aussi, le travail résumé dans ce chapitre avait pour objectifs d'étudier les réductions dans le sol en anaérobiose de FeIII et de MnIV, et leur interdépendance avec les autres transformations biogéochimiques en se focalisant sur (i) les contributions relatives des réductions microbiennes directes et abiotiques à ces réductions, (ii) pour les réductions microbiennes directes, l'impact de différents types de donneurs d'électrons (H₂, acides organiques, sucres) et accepteurs d'électrons (NO₃⁻, SO₄²⁻), et (iii) la physiologie des réducteurs de FeIII (aptitude à croître sans réduction de FeIII ou à utiliser d'autres voies cataboliques (dénitrification, sulfato-réduction, fermentations...)).

Nos expériences comportaient des incubations anaérobies en batch de Ferralsol (FAO classification) amendé avec de la vinasse de rhumerie durant 0 (V₀), 2 (V₂) et 7 j (V₇) d'anaérobiose, suivis de 2 j d'anaérobiose après apport d'un donneur d'électrons (glucose,

lactate ou H₂), voire simultanément d'un accepteur d'électrons (NO₃⁻ ou SO₄²⁻). Les analyses ont inclus (i) la caractérisation des gaz, des solutés et du fer en phases solides, et (ii) des dénombrements des bactéries par groupes fonctionnels, en déterminant les proportions des réducteurs du FeIII à effectuer d'autres catabolismes.

De manière similaire aux 1^{ères} expériences, les fermentations et l'acétogénèse réductrice de protons ont expliqué la totalité des dégradations des composés organiques au cours de la première phase d'incubation des traitements V₂ et V₇, suggérant que la réduction du FeIII et le MnIV s'effectuait majoritairement *via* l'oxydation de H₂ ou de précurseurs de H₂. Cependant au cours de cette première phase d'incubation, l'évolution en nombre des réducteurs du FeIII est apparue bien faible au regard de la biomasse qui pouvait être produite grâce à l'énergie récupérée au cours de la réduction de FeIII. De plus au cours de la deuxième phase d'incubation, la dynamique des réducteurs de FeIII a fait apparaître parfois des évolutions (baisse ou augmentation) en "opposition" avec les quantités de fer réduit en solution (augmentation ou baisse). Ces expériences indiqueraient donc soit une réduction microbienne sans gain d'énergie par d'autres micro-organismes (fermentaires probablement, sulfato-réducteurs éventuellement), soit une réduction abiotique associée à la production microbienne d'autres composés réduits pouvant servir de réducteurs pour FeIII ou MnIV. Ces expériences ont également confirmé la capacité des réducteurs de FeIII à utiliser d'autres voies cataboliques. Tous les réducteurs de FeIII étaient sulfato-réducteurs ; moins de 10 % étaient réducteurs du NO₃⁻ ou fermentaires, à l'exception des caractérisations en fin d'incubation après apport de glucose ou de lactate. Aussi, leur dynamique et leur diversité fonctionnelle dépend-t-elle de l'expression de leurs autres fonctionnalités en fonction des conditions de milieu. L'apport de NO₃⁻ a eu un effet dépressif sur les activités et sur la croissance des bactéries réductrices du FeIII. Ces phénomènes peuvent être attribués d'une part à un effet toxique sur les réducteurs de FeIII de NO₂⁻ produit lors de la réduction du NO₃⁻, et d'autre part aux réactions abiotiques entre NO₃⁻ et FeII en solution et/ou en phase solide.

Ce chapitre 4 correspond à un article qui sera soumis à la revue Soil Biology and Biochemistry avec pour titre "Mobilisation of Fe and Mn with regard to the dynamics of FeIII reducers in an anaerobic soil slurry supplied with rum vinasse" et pour auteurs : N. Pautremat, P. Renault, P. Cazevielle, J.C. Jumas and M.L. Fardeau.

Mobilisation of Fe and Mn with regard to the dynamics of FeIII reducers in an anaerobic soil slurry supplied with rum vinasse

Abstract

Spreading liquid organic wastes on soil enhances the risk of mobilisation of metals under anaerobic conditions. A better understanding of links between FeIII and MnIV reduction and FeIII reducers' dynamics requires researches on (i) the interactions between FeIII reducers and other microbial communities, (ii) the impact of electron donors/acceptors on biotransformations and FeIII reducer dynamic, and finally (iii) the characterisation of possible contribution of abiotic reactions to FeIII reduction. In our experiments, a Ferralsol (FAO classification) was supplied with a vinasse and anaerobically incubated during either 0 (V₀), 2 (V₂) or 7 days (V₇), and then anaerobic incubation were carried out after the addition of electron donors (glucose, lactate, H₂) and electron acceptors (NO₃⁻, SO₄²⁻) for 2 more days. Analyse included (i) characterisations of gases, solutes and solids, (ii) microbial enumerations by functional groups and proportion of FeIII reducers with the capacity to other catabolism. During V₂ and V₇ incubations, fermentations and true acetogenesis explained most of organic compound degradation, suggesting that reduction of FeIII and MnIV was mainly coupled with H₂ oxidation. However, FeIII reducers' dynamic appeared low with regard of biomass estimation based on FeIII reduction during the first phase of incubation. Moreover, after the addition of substrates, growth of FeIII reducers was not systematically correlated to FeIII reduction. These observations suggest that other communities contributed indirectly to FeIII reduction, by reduction without gaining energy by other bacteria communities or by abiotic reduction due to the microbial production of compounds susceptible to reduce FeIII and MnIV. These experiments confirmed the capacity of FeIII reducers to use other metabolites. In all treatments, FeIII reducers were SO₄²⁻-reducers, less than 10 % were able to denitrify or to ferment, at the exception of treatments with glucose and lactate addition. So FeIII reducers' dynamic and functional diversity depend on the expression of their other functions according to the medium conditions. At last, NO₃⁻ had a depressive effect on microbial growth and FeIII reduction. This could have resulted simultaneously from NO₂⁻ toxicity on FeIII reducers, and either possible abiotic re-oxidation of FeII with the concomitant reduction of NO₃⁻. For all treatments and dates, FeIII reducers could reduce SO₄²⁻.

IV.1 Introduction

Human activities generate liquid wastes (EC, 2001), from which the spreading supplies the soil with nutrients, water and organic matter, and can positively affect plant yield (Nnabude and Mbagwu, 2001) and soil structure (Angin et al., 2005). However, wastes often contain heavy metals (Speir et al., 2003), organic xenobiotics (Angelidaki et al., 2000), pathogens (Pell, 1997) and/or concentrated salts (Gemtos et al., 1999). In addition, the concomitant supply of water and organic compounds enhances the risk of anaerobiosis (Quantin et al., 2001) and favours the emission of greenhouse gases (Conrad, 1996), the production of toxic compounds (Roy and Conrad, 1999; Aguilar et al., 1995; Visser et al., 1997) and the mobilisation of metals (Quantin et al., 2001). Metal mobilisation can have toxicological effects on plants (Hue et al., 2001) and affect human health (Doran and Zeiss, 2000).

Until recently, metal reductions have been regarded as abiotic reactions only (Münch et Ottow, 1983), and some experiments have confirmed that abiotic processes could prevail (e.g. the oxidation of HS^- into S^0 coupled with the reduction of FeIII (Murase and Kimura, 1997)). Similarly, some reduced metals may be oxidized abiotically in anoxic conditions (e.g. the oxidation of green rust FeII with the concomitant reduction of NO_3^- into NH_4^+ (Hansen et al., 1996)). However in the late 1980s, micro-organisms that can reduce FeIII or MnIV with the oxidation of organic compounds were isolated, and grown under these conditions (Lonergan et al., 1996). It is now recognized that Fe and Mn mobilisations occur mainly by direct microbial reduction of FeIII and MnIV contained in oxy-hydroxides (Lovley et al., 1989; Nealson and Little, 1997; Glasauer et al., 2003; Lloyd, 2003; Bonneville et al., 2004) and clays (Favre et al., 2002; Kostka et al., 2002). Microbial reduction of metals can proceed by either direct contact between soil particles and microbial cells (Das and Caccavo, 2000), soluble electron shuttles (e.g. humic substances, quinones) (Nevin and Lovley, 2002; Loyd, 2003), or complexation or chelation of metals that enhance their mobilisation before their reduction (Kendall and Hochella, 2003). Environmental conditions affect metal reduction. Firstly, the minerals themselves affect the energy yield of the reaction (Kostka and Nealson, 1995), the kinetic of the reaction (Roden and Zachara, 1996) and the nature of the produced solids (Ona-Nguéma, 2003). Secondly, the nature and concentration of electron donors (Lovley et al., 1989) also affect the reductions; they include fatty acids (Lovley and Phillips, 1988), carbohydrates, polyols, amino-acids (Coates et al., 1999), monoaromatic compounds

such as benzoate, phenols and toluene (Lovley and Lonergan, 1990) and H₂ (Coppi et al., 2004). Metal reducers involve a broad functional diversity of bacteria (Lonergan et al., 1996). FeIII and MnIV reducers can commonly also grow with UVI, CrVI, SeVI PbII, TcVII and HgII as terminal electron acceptors (Lovley, 1995). Moreover, several of them can reduce other electron acceptors such as fumarate, NO₃⁻ and S⁰, SO₄²⁻ (Lonergan et al., 1996). Similarly, many fermentative micro-organisms can grow with FeIII or MnIV as minor electron acceptor during fermentation (Lovley, 1991), and some SO₄²⁻ reducing bacteria can reduce FeIII and MnIV (Coleman et al., 1993).

The interrelations between soil functional microbial diversity and metal reduction are still questionable with (i) the relative contributions of direct microbial FeIII and MnIV reductions *versus* indirect FeIII and MnIV reductions since FeIII and MnIV reductions can result from the combination on microbial processes and abiotic ones (Murase and Kimura, 1997), (ii) the possibility for FeIII reducers to use other catabolic pathways and then the relative contribution of these pathways to the dynamics of metal reducers, and (iii) the preferential use or not of H₂ as electron donor in metal respiration. Methodological limits dealing with the enumeration of metal reducers (Lovley, 1991) and the measure of FeII and MnII in solids have made it difficult to work on these questions. Recent improvements (Geesey et al., 2002) have enabled the estimation of ⁵⁷FeII to ⁵⁷FeIII ratio by Mössbauer spectroscopy (McCamonn, 1995), and the FeII to FeIII and MnII to MnIV ratio by EXAFS (Spadini et al, 2003; Friedl et al., 1997) in solids. In contrast, there is still a need to develop new tools for microbial enumeration, molecular diversity and physiology characterization (Hill et al., 2000).

The aims of this work were to (i) follow under anaerobic conditions the dynamic of FeIII reducers and other functional communities in a soil supplied with vinasse, and (ii) attempt to relate metal reduction and mobilisation (Fe and Mn) to the FeIII reducer density and dynamics, accounting for the ability of several FeIII reducers to denitrify, reduce SO₄²⁻ and/or ferment.

IV.2 Materiels and Methods

IV.2.1 Soil and organic liquid waste

Experiments were performed on a Ferralsol sampled in Reunion Island at "La Mare" experimental station, 20°40' S and 55°32' E. The annual average precipitation is about 1500 mm with a potential evapotranspiration of 2300 mm. Sampling was performed on the 0-

20 cm layer of the soil on the 3rd December 2002 after sugarcane harvest. The soil was then air-dried (9.67 % residual moisture), stored in hermetic bags, and flown to Montpellier under cold conditions. It was mechanically crushed, sieved at 2 mm, and stored again in plastic bags at 4°C until the beginning of the experiments. Its properties were as follows: 679 g kg⁻¹ clay; 278 g kg⁻¹ silt; 44 g kg⁻¹ sand; 19.9 g kg⁻¹ organic C; 1.59 g kg⁻¹ organic N; 1.74 mg kg⁻¹ N-NH₄⁺; 6.16 mg kg⁻¹ N-NO₃⁻; 144.15 g kg⁻¹ total Fe, 3.20 g kg⁻¹ total Mn, 0.108 g kg⁻¹ total Cr, 0.963 g kg⁻¹ total Ni. Mobilised metals by DTPA extraction were: 24.78 mg kg⁻¹ Fe, 107.9 mg kg⁻¹ Mn, 0.40 mg kg⁻¹ Ni and Cr at concentration lower than 0.10 mg kg⁻¹. No FeII was detected in the solid phases of this soil.

The rum vinasse from molasses of sugarcane was collected at the low exit of a distillation column on the 10th December 2002. It was stored at -20°C until experiments began. Dissolved Organic Carbon of the vinasse was 1.83 mol C l⁻¹ and N concentration of alpha amino acid after HCl (6N) hydrolysis was 1.80 10⁻² mol N l⁻¹. Vinasse contained 4.92 10⁻¹ mol C l⁻¹ of total carbohydrates, 4.62 10⁻² mol C l⁻¹ or 1.22 10⁻² mol N l⁻¹ total proteins, 1.05 10⁻¹ mol C l⁻¹ total phenols and other soluble organic matter including polyols, small organic acids, phenols and alcohols. The composition in main small carbohydrates, polyols, organic acids and alcohols was measured by Ionic Chromatography (IC) (cf. Table 1 section III.2.1.). The vinasse pH was 4.58.

IV.2.2 Experimental procedures

In order to follow the reduction and mobilisation of FeIII and MnIV, and attempt to relate these processes to catabolic activities and dynamics of microorganisms, the soil was supplied with the vinasse and anaerobically incubated during either 0, 2 or 7 days. The amount of supplied vinasse varied with the time of incubation and was a compromise between the satisfaction of maximal activity requirements and the disappearance of easily used carbohydrates at the end of incubations. After these incubations, analyses and additional incubations were performed. Analyses included:

- i/ the characterisations of gases, solutes and solids in order to assess the importance of the main anaerobic catabolic pathways among NO₃⁻ reduction, fermentations and true acetogenesis, FeIII and/or MnIV reductions, and SO₄²⁻ reduction, since CH₄ production can be neglected for these short periods (Section III);

Table 1: Planning of measurements at the end of each treatment incubation

| Treatments | Microbiology | Liquid and gas phases (3 flasks repetitions) | Solid phase |
|-------------------|---------------------------------|---|---|
| V ₀ | BFG* & IRB* (2 replicates) | Small carbohydrates, Organic Acids, Polyols, Free Amino Acids SO ₄ ²⁻ , PO ₄ ³⁻ , NO ₃ ⁻ , NO ₂ ⁻ , NH ₄ ⁺ , Total NH ₄ ⁺ , Fe _{tot} , Mn _{tot} , Cr _{tot} , Ni _{tot} , FeII | Mössbauer FeII/FeIII ratio (1 sample) |
| V _{0-C} | IRB* | E _H , pH, gases | ✓ |
| V _{0-L} | (3 replicates) | | ✓ |
| V _{0-LN} | | | ✓ |
| V ₂ | BFG* & FeIII RB* (2 replicates) | | ✓ |
| V _{2-C} | | | ✓ |
| V _{2-G} | | | ✓ |
| V _{2-H} | IRB* | | ✓ |
| V _{2-L} | (3 replicates) | | ✓ |
| V _{2-LN} | | | ✓ |
| V _{2-LS} | | | ✓ |
| V ₇ | BFG* & IRB* (2 replicates) | | ✓ |
| V _{7-C} | IRB* | | ✓ |
| V _{7-L} | (3 replicates) | | ✓ |
| V _{7-LN} | | | ✓ |

BFG: enumeration of bacteria by functional group and total anaerobes, IRB: enumeration of the FeIII reducers and determination of their others metabolisms

- ii/ microbial enumerations (anaerobes, NO_3^- reducers, fermentative bacteria, FeIII reducers, SO_4^{2-} reducers), and the proportion of FeIII reducers that are also either NO_3^- reducers, fermentative bacteria or SO_4^{2-} reducers.

Additional incubations were performed to check the FeIII reducing behaviour of the incubated soil slurry to the addition of an electron donor (glucose, lactate or H_2), and sometimes an electron acceptor (NO_3^- or SO_4^{2-}).

IV.2.2.1 Treatments and measurements

Batch incubations were performed in sealed 150 ml flasks. Seven ml of milliQ water were added to 40 g of soil at the conservation moisture in individual flasks 7 d before the beginning of the incubations. Just before the beginning of the incubations, 3 treatments were then prepared: V_0 , V_2 and V_7 , corresponding to incubation duration: 0 (anaerobic set up), 2 and 7 days, respectively:

- V_0 : 47 g of humidified soil + 60 ml of milliQ water;
- V_2 : 47 g of humidified soil + 60 ml of 1:30 diluted vinasse;
- V_7 : 47 g of humidified soil + 60 ml of 1:8 diluted vinasse.

All the slurries were put in anaerobic conditions, by alternating 3 successive cycles of 10 min vacuum and 10 min N_2 replacement. The last N_2 filling of the flask was considered as the initial time of the incubation. Flasks were set up to atmospheric pressure. The flasks were then incubated at 25°C in darkness and continually shaken (150 rpm) (Cooled Orbital Incubator, Gallenkamp, U.K.). At the end of the incubations, measurements on the solid, liquid and gaseous phases as well as bacteria enumerations were performed as described in [Table 1](#). t_0 , t_2 and t_7 corresponded to date at the end of anaerobic phases of V_0 , V_2 and V_7 treatments, respectively. For microbial enumeration, a date of analysis was added after 2 d of anaerobiosis for V_7 treatment; it was determined as t_2' .

For the V_0 treatment and at the end of V_2 and V_7 incubations, substrates were added to the slurries through the stoppers (with a 1 ml syringe) and the atmosphere of the flasks were instantaneously replaced by 100 % N_2 atmosphere; except for the V_{2-H} treatment, in which the atmosphere was replaced by H_2 . Gas measurements ensured no O_2 and only traces of CO_2 at the beginning of this second incubation period. Substrates were added to the slurry by the addition of 1 ml of the following solutions in the flasks:

- V_{x-C} : milliQ water;

- V_{x-G} : 61.25 g glucose l^{-1} solution;
- V_{x-H} : milliQ water and replacement of the flask atmosphere with H_2 ;
- V_{x-L} : 60.96 g sodium lactate l^{-1} solution;
- V_{x-LN} : 60.96 g sodium lactate l^{-1} and 165.0 g KNO_3 l^{-1} solution;
- V_{x-LS} : 60.96 g sodium lactate l^{-1} and 165.0 g Na_2SO_4 l^{-1} solution;

where x is an index for the duration of the first incubation period (i.e. with vinasse). [Table 1](#) lists the performed treatments. After addition of the substrate(s), incubation was carried out for 2 additional days. Additional analyses on the liquid phase were performed after 1 d of incubation in order to assess whether substrate consumption and product accumulation were linear or not. [Table 1](#) describes the analyses performed at the end of the second phase of incubation.

IV.2.2.2 Analyses on liquid, gaseous and solid phases

Gas measurements (O_2 , CO_2 , N_2 , N_2O , H_2) were performed on a TCD gas chromatograph (HP 5890 Series II, Hewlett Packard, USA) fitted with Porapak Q (80-100 μm mesh, 1.8 m) and sieve molecular (1-5 Å, 1.8 m) columns coupled to a PC workstation with an integration software. Carrier gas was He. Relative uncertainties were about 1% (3% for H_2). Before gas sampling, the flasks were vigorously shaken to ensure equilibrium between gas and liquid phases. 0.20 ml of gas was then sampled with a syringe and analysed by TCD chromatography.

To prevent oxidation of the media and pH variation due to CO_2 volatilisation, the pH and the redox potential E_H were measured as soon as the flasks were opened under N_2 atmosphere in a glove bag using pH and E_H electrodes (933327 and 90437, respectively, Bioblock Scientific, France). The measurements were performed on the supernatant.

Triplicate flasks for each date of analysis were specifically used for a KCl extraction of NH_4^+ that was performed with soil to KCl solution (1 M) weight ratio equivalent to 1:2.5.

For all flasks, the supernatant sampling was performed under strict N_2 atmosphere, as well as the centrifugation that was operated during 15 min at 1800 g at 4°C (Heraeus, Biofuse stratus, Germany), the filtration at 0.22 μm (Millex GP, Millipore, France), and the distribution of the solution in sterile tubes. The measurement of FeII concentration was immediately done. Tubes intended for the measurement of organic compounds were supplied with NaN_3 to obtain 30 mg l^{-1} concentration. Tubes intended to measure total metal

concentrations were supplied with 25 μ l concentrated HCl in order to avoid oxy-hydroxide precipitation. No chemical was added to the tubes intended for N-NO_3^- , N-NO_2^- and N-NH_4^+ analyses. All the tubes were then stored at 4°C until measurements.

Colorimetric method (Jasco V-530, UV-VIS Spectrophotometer, Tokyo, Japan) were used for the determination of total carbohydrates at 627 nm (Dubois et al., 1956).

The concentrations in small organic compounds were determined by IC (Dionex DX 600, U.S.A.) fitted with (i) both CARBOPAQ-PA1 and CARBOPAQ-MA1 columns with a detector of pulsed amperometry for sugars and polyols, (ii) a CARBOPAQ-MA1 column and a detector of pulsed amperometry for alcohols, and (iii) an IONPAQ-AS11 column and a conductimetric detector for organic acids and mineral anions, such as NO_3^- , SO_4^{2-} , Cl^- and PO_4^{3-} . However, we have retained measurements of NO_3^- , NO_2^- and NH_4^+ performed by spectroscopy (Alliance, EvolutionII, France).

Total concentrations of Fe and Mn in solution were determined by ICP-AES (Varian, VISTA-Pro, CCDsimultaneous, France), whereas DPKBH was used as a reagent to determine FeII concentration by spectrophotometry at 660 nm (Bourri  et al., 1999).

^{57}Fe M ssbauer measurements were performed at 75 K with an EG and G constant acceleration spectrometer in transmission mode. The γ source of nominal activity 10 mCi was ^{57}Co in a Rh matrix. The velocity scale (± 10 mm/s) was calibrated with reference to the magnetic sextet spectrum of a metallic iron foil absorber and all isomer shifts are given with respect to the centre of this spectrum. After incubation and additional treatments the absorbers were prepared inside a glove bag under N_2 atmosphere, from approximately 500 mg of samples and introduced in specific lead sample holder (10 mm diameter, 2 mm thick), tight and transparent to the γ rays. This cell was composed of 2 very thin 14 mm diameter Mylar windows separated by 2 mm of lead. Experimental data were analysed using the ISO software (K nding, 1969) by fitting the recorded spectra to Lorentzian profiles by the least squares method. Goodness of fit was controlled by the classical “chi-squared” test. Due to probable evolutions of solid phase between sampling and analysis, we took only into account FeII in solid phase at the end of V_7 and $\text{V}_{2\text{-LN}}$ treatments.

De nouvelles analyses par spectroscopie M ssbauer seront effectu es avant soumission de l'article.

Table 2: Selective culture media for general anaerobes and bacteria by functional groups and reagents of specific metabolites production.

| Metabolic type | Medium preparation (g l ⁻¹) | Reagent | References |
|--|--|---------------------------|---------------------------------|
| Anaerobes | TrypticSoy Broth prepared under anaerobic conditions | Turbidity | Küsel et al., 1999 |
| Fermenters | NH ₄ Cl, 1 ; KH ₂ PO ₄ , 0.3 ; K ₂ HPO ₄ , 0.3, CaCl ₂ , 0.1 ; KCl, 0.1; NaCl, 1; cysteine | Turbidity | Ben Dhia Thabet et al., 2004 |
| | HCl.H ₂ O, 0.5 ; yeast extract, 1 ; trace element solution ^a , 10ml l ⁻¹ , 5 ml l ⁻¹ rezaurine, 0.001; Na ₂ S.9H ₂ O, 0.4; NaHCO ₃ , 2; MgCl ₂ , 3; glucose, 3.6, peptone, 4. | | |
| Denitrifiers | Nutrient Broth 2X; 10 ml KNO ₃ (5mM); trace element solution, 1 ml l ⁻¹ , vitamin solution, 1 ml l ⁻¹ | Griess-Illosway's reagent | Lensi et al., 1995 |
| | | Morgan's reagent | |
| SO ₄ ²⁻ reducers | NH ₄ Cl, 1 ; KH ₂ PO ₄ , 0.3 ; K ₂ HPO ₄ , 0.3, CaCl ₂ , 0.1 ; KCl, 0.1; NaCl, 1; cysteine | CuSO ₄ | Vester and Ingvorsen, 1998 |
| | HCl.H ₂ O, 0.5 ; Na ₂ SO ₄ , 4 ; yeast extract, 1 ; trace element solution ^b , 1ml l ⁻¹ , vitamin solution, 5 ml l ⁻¹ rezaurine, 0.001; Na ₂ S.9H ₂ O, 0.4; NaHCO ₃ , 2; MgCl ₂ , 3; lactate, 20mM | | |
| FeIII reducers | NaHCO ₃ , 5.56; CaCl ₂ , 0.22; KCl, 0.22; NH ₄ Cl, 3.34; NaH ₂ PO ₄ , 0.8; lactate, 2.22; acetate, 2.22; pyruvate: 2.22; ferric citrate: 4.9; trace element solution ^c , 1 ml l ⁻¹ ; vitamin solution, 1 ml l ⁻¹ | DPKBH | SSSA, 1994 |
| | | | |

Trace element solution: (a) Balch trace element solution without sulfates (Balch et al., 1979), (b) Widdel trace element solution (Widdel and Penning, 1981), (c) trace element solution (SSSA, 1994)

Vitamin solution: (Wolin et al., 1963)

Bacteria were enumerated by the most probable number (MPN) technique with selective media (Table 2) for some microbial functional groups. 5 ml aliquots of these selective media were initially dispensed in sterile hungate tubes and sealed with sterile rubber stopper, the atmosphere containing 100 % N₂. The hungate tubes were autoclaved at 120°C for 15 min, and strict anaerobic and aseptic conditions were maintained throughout the experiments (Alef, 1995). Sterilised vitamin solution and sugar solution were added thereafter just before inoculation, without adding O₂.

The successive dilutions of the soil slurry were obtained as follow. Firstly, 12 ml of 133.3 g NaCl l⁻¹ solution were added to each flask (dilution 0), in order to obtain an initial slurry solution at approximately 0.8 % NaCl, and the flasks were shaken for 1 h to disperse the micro-organisms. Successive 5-fold dilutions were thereafter obtained in 0.8 % NaCl solutions maintained in anaerobic and sterile conditions. Inoculations were finally performed by supplying 0.5 ml diluted samples in the hungate tubes containing 5 ml of the selective media; 8 replicates for each dilution series were done. All the tubes were then incubated at 28°C for about 10 d. For each MPN series, 3 non inoculated tubes were added as controls for medium sterility. After about 10 d of incubation, for 'total' anaerobes and fermentative bacteria, tubes were scored positive when microbial growth was observed (turbid suspension, bacterial clumps or filaments on the culture tube wall). For NO₃⁻ reducers, SO₄²⁻ reducers and FeIII reducers, tubes were scored positive when either substrates specific to the functional community disappeared or products also specific to the functional community accumulated. Microbial functional enumerations and confidence levels were determined using statistical MPN tables (Cochran, 1950). Differences between treatments were considered significant for deviation in values higher than 3 times the MPN determined value.

Successively to the enumeration of FeIII reducers, all positive tubes of the 5 last dilutions were considered. After manual stirring, 0.5 ml of each of the solution of these hungate tubes were sampled and added, under strict anaerobic and sterile conditions, to hungate tubes previously filled with 5 ml of selective media for other microbial activities (NO₃⁻ reduction, fermentation or SO₄²⁻ reduction). All tubes were then incubated at 28 °C for about 10 d. Non inoculated tubes were included as controls for medium sterility. MPNs were scored positive as previously. Apparent proportions of FeIII reducers that were also either NO₃⁻ reducers, SO₄²⁻ reducers or fermentative bacteria were determined by the percentage of positive tubes

after re-inoculation on specific media above the numbers of positive tubes of the 5 last considerate dilutions for the FeIII reducers MPN. These apparent proportions P_{A-i} (were i is for either NO_3^- reduction, SO_4^{2-} reduction or fermentation) could be estimated from the actual proportions p_{R-i} using an empirical function that was fitted to several punctual calculations at various microbial density d_F of the 4th dilution (generally, the intermediate dilution among the 3 ones for which neither all the tube replicates or none of these tubes were positive or negative):

$$P_{A-i} = \frac{p_{R-i}^{\left(\frac{a+b}{d_F^{0.2}}\right)}}{p_{R-i}^{\left(\frac{a+b}{d_F^{0.2}}\right)} + \left(\left(c \times e^{\left(\frac{d_F-0.15}{d_F+e}\right)} + (1-c) \right) \times \left(1 - p_{R-i}^{\left(\frac{a+b}{d_F^{0.2}}\right)} \right) \right)} \quad (1)$$

It enables us to estimate unbiased proportions p_{R-i} of FeIII reducers that were also NO_3^- reducers, SO_4^{2-} reducers or fermentative bacteria:

$$p_{R-i} = \left(\frac{P_{A-i} \times \left(c \times e^{\left(\frac{d_F-0.15}{d_F+e}\right)} + (1-c) \right)}{1 - P_{A-i} + \left(P_{A-i} \times \left(c \times e^{\left(\frac{d_F-0.15}{d_F+e}\right)} + (1-c) \right) \right)} \right)^{\left(\frac{1}{\left(\frac{a+b}{d_F^{0.2}}\right)}\right)} \quad (2)$$

where a, b, c, d are empirical constants, equal to -0.170, 0.695, 0.612, 1.051, respectively.

IV.2.3 Data treatments

IV.2.3.1 Mass balance calculations

For mass balance calculations, compound concentrations were determined from solution concentration corrected by taking into account adsorption of supplied compounds. Unpublished experimental results enabled us to estimate retention coefficients for several small organic compounds as reported in [table 3](#).

As long as acetic and butyric fermentations, as well as true acetogenic transformations explained most of acetate, ethanol and butyrate fates, the index I_f (mol C) corresponds to the amount of glucose C required to produce butyrate, acetate and ethanol:

Table 3: Retention coefficients for supplied organic compounds on Ferralsol.

| Organic Compounds (by pool or individual) | Retention coefficient | Organic Compounds (by pool or individual) | Retention coefficient | Organic Compounds (by pool or individual) | Retention coefficient |
|--|--------------------------|--|--------------------------|--|--------------------------|
| COD | 0.42 | \sum <i>Organic Acids</i> | 0.54 | \sum <i>Polyols</i> | 0.17 |
| Total carbohydrates | 0.26 | Acetate | 0.26 | Arabitol | 0.08 |
| Total proteins | 0.79 | Butyrate* | - | Glycerol | 0.27 |
| Total phenols | 0.38 | Citrate | 1.00 | Inositol | 0.01 |
| | | Formate | 0.37 | Mannitol | -0.08 |
| \sum <i>Identified small sugars</i> | -0.66 | Isoaconitate | 0.88 | Sorbitol | -0.05 |
| Arabinose | -0.65 | Lactate | 0.58 | | |
| Fructose | 0.24 | Malate | 0.50 | \sum <i>Alcohols</i> | 0.44 |
| Galactose* | - | Oxalate | 0.55 | Ethanol* | - |
| Glucose | -2.53 | Propionate | -0.65 | Isopropanol | 0.48 |
| Mannose* | - | Pyruvate | 0.70 | | |
| Saccharose | 0.163 | Tartrate | 0.55 | | |
| Ribose* | - | Transaconitate | 0.79 | | |
| Trehalose | 0.329 | | | | |

* Compounds detected in the slurry at 0.5 h, but not detected in the added vinasse.

** Retention coefficient (k_i) were determined $Q_i(\text{total})=Q_i(\text{solution})/(1-k_i)$, where $Q_i(\text{solution})$ and $Q_i(\text{total})$ were total and solutes amounts of compound I (molC). Value < 0 corresponding to bacteria excretions

$$I_f = 6 \times \left\{ (\Delta Q_b/4) + \frac{((\Delta Q_a/2) + (\Delta Q_e/2))}{2} \right\} \quad (3)$$

where ΔQ_b , ΔQ_a and ΔQ_e are the variations (mol C) for a given period of butyrate, acetate and ethanol, respectively. This index has been decreased to account for acetate production from anaerobic oxidation of other organic acids, assuming no by-products (e.g. CO₂):

$$I_{rf} = I_f - \left(\frac{3}{2} \times \Delta Q_{OA} \right) \quad (4)$$

where I_{rf} is in mol C, and ΔQ_{OA} is the variation of other organic acids (mol C). Similarly, we may define an index I_{fm} (mol C) that represents the amount of glucose C required to produce CO₂ by acetic and butyric fermentations with formate lysis or oxidation:

$$I_{fm} = 3 \times \Delta Q_{CO_2} \quad (5)$$

where ΔQ_{CO_2} is the variation in the amount of total CO₂ (mol), for a given period, including gas and solutes (H₂CO₃ and HCO₃⁻). Additional indexes I_{fc} and I_{fc+p} (mol C) were defined for the variations in 'carbohydrates' and 'carbohydrates+polyols' pools. In addition, we indirectly assess the maximum gross H₂ production Q_{H_2} (mol H₂) through fermentation (i.e. due to formate lysis) and true acetogenesis:

$$Q_{H_2} = \frac{2}{3} \times I_{rf} + \Delta Q_i(\text{formate}) \quad (6)$$

where $\Delta Q_i(\text{formate})$ is the variation in the amount of formate (mol C). This production is lowered to account for (i) maximum formate oxidation with NO₃⁻ reduction, and (ii) SO₄²⁻ reduction that can either lower H₂ production or consume H₂:

$$Q_{m-H_2} = Q_{H_2} - (\Delta Q_{NO_3^-} + 4 \times \Delta Q_{SO_4^{2-}}) \quad (7)$$

As long as homoacetogenesis and hydrogenoclastic methanogenesis can be neglected, either 2 x Q_{m-H_2} mol of FeIII could be reduced into FeII, or Q_{m-H_2} mol of MnIV into MnII, and Q_{m-H_2} was compared with the I_{aq-M} index (mol H₂) for the mobilisation of metals:

$$I_{aq-M} = \frac{Q_{aq}(\text{FeII})}{2} + Q_{aq}(\text{MnII}) \quad (8)$$

The I_{aq-M} -to- Q_{m-H_2} ratio was an estimate of the proportion of reduced metals that are really mobilised, as long as Q_{m-H_2} is a good estimate of H₂ really available for metal reduction.

IV.2.3.2 Activity notations

After the first incubation period and the addition of electron donors or/and acceptors to V₀, V₂ and V₇ treatments, we qualified the metabolic pathways during the second incubation period using the electron balances involved in each pathway. We assumed that the fermentation of 1 mol of hexose (that can produce 4 mol H₂), corresponded to a flux of 8 mol of electron (i.e. 4/3 mol of electron per mol C hexose). Similarly, we assumed that 1 mol of NO₃⁻ and SO₄²⁻ corresponded to fluxes of 8 mol electron, and that the reduction of 1 mol FeIII and 1 mol MnIV corresponded to the production of 1 and 2 mol electron, respectively.

IV.2.3.3 Population dynamics and biomass calculations

The increase in biomass of each of the microbial functional types (NO₃⁻ reducers, fermentative bacteria, FeIII reducers, SO₄²⁻ reducers) were calculated assuming that (i) the dynamics of the microbial types depends only on their defining activity (ii) 1 mol ATP enables the production of 6.5 and 12 g biomass for fermentative bacteria and other microbial types, respectively, and (iii) the following energetic yields per reaction:

- 17/3 mol ATP per mol of hexose consumed through fermentation (Pelmont, 1993);
- 95/24 mol ATP per mol of NO₃⁻ reduced (Stouthamer, 1988);
- 2 mol ATP per mol of SO₄²⁻ reduced (Widdel, 1988; Pelmont, 1993);
- 2/3 mol ATP per mol of FeIII reduced, considering either only the solution or simultaneously the solid and liquid phases (Lovley and Chapelle, 1995).

Biomass increase was then converted in cell number assuming a specific bacteria mass of about 2.7 10⁻¹³ g bacteria⁻¹ (Reynolds and Pepper, 2000).

IV.2.3.4 Metal speciation

Data treatments aimed to (i) assess the contribution of mineral and organic ligands, which are involved in microbial activities as substrates or products, to metal complexation and mobilisation, and (ii) check whether FeII and MnII could precipitate and identify the minerals that could thermodynamically exist. Chemical speciations in solution were calculated using GEOCHEM-PC Geochemical model (Parker et al., 1995). The database of equilibrium

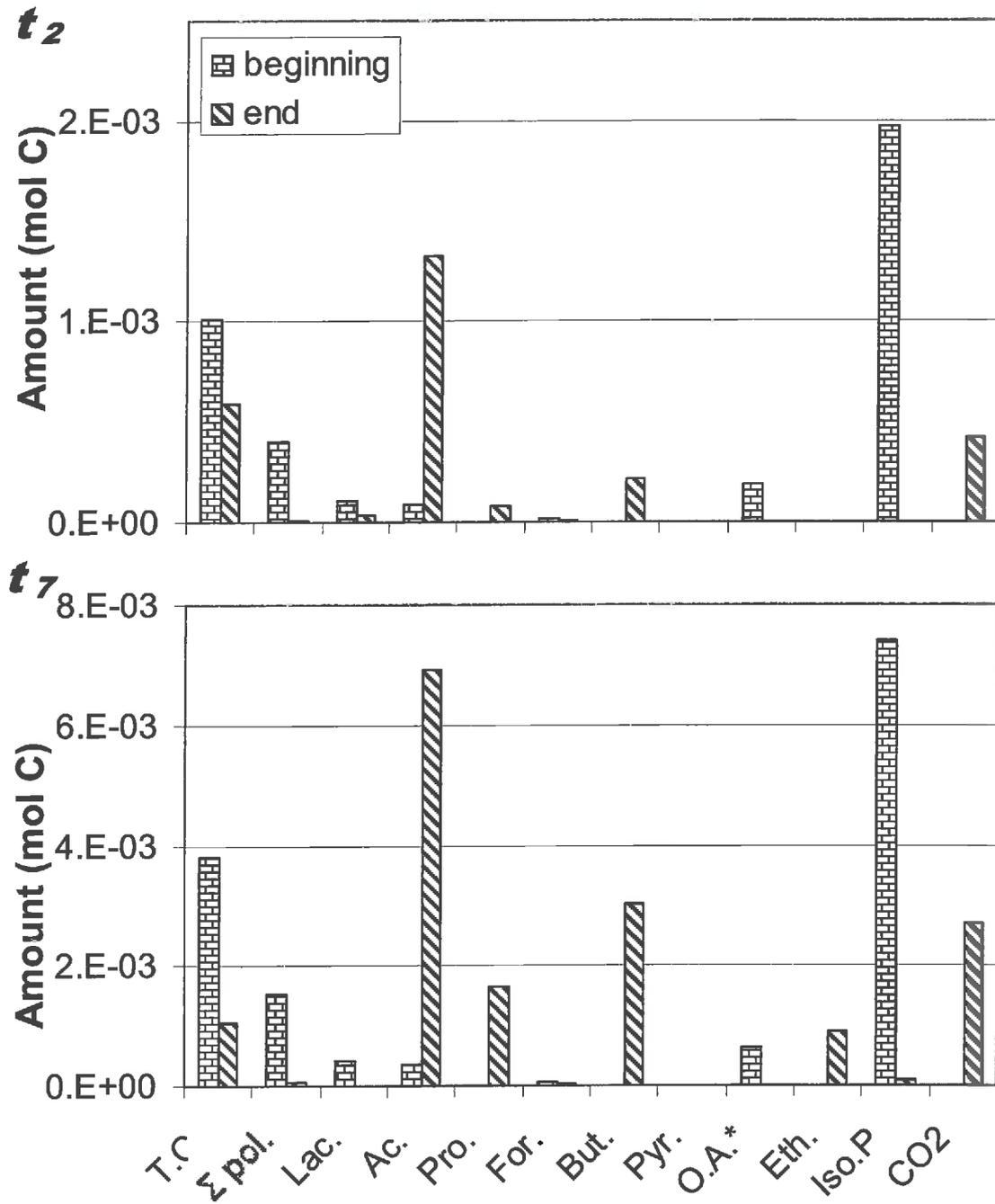
constants combined the database of [Parker et al. \(1995\)](#) and additional constants issued from the NIST standard reference database 46 version 7.0 for the following ligands: propionate, lactate, butyrate, pyruvate, cisaconitate, transaconitate, tryptophan and asparagine. In addition, saturation indices (SI) for potentially precipitating mineral phases were calculated using solution data and compared with the corresponding pKs values found in literature ([Stumm and Morgan, 1996](#); [Génin et al., 1998](#)). Saturation indices, for magnetite, carbonated green rust, chloride green rust, sulfated green rust, siderite, rhodocrosite, alabandite, pyrite, FeII and MnII-hydroxides, were calculated in soil solution at t_2 of V_2 and t_7 V_7 , and at the end of incubation after addition of substrates to the slurries.

IV.3 Results

IV.3.1 Geochemical evolutions during the initial anaerobic incubations

During the 2 days of the initial anaerobic incubation of the V_2 treatment, total carbohydrate, small sugar (C_5 , C_6 , C_{12}) and polyol concentrations decreased by 77.4, 83.0 and 97.0 %, respectively ([Fig. 1](#)). At t_2 , most of small organic acids were consumed, except acetate, butyrate, formate, lactate and propionate. Along the 2 d of anaerobiosis, lactate decreased by 64.1 % ($\Delta = 1.29 \cdot 10^{-5}$ mol flask $^{-1}$), whereas produced propionate was $8.01 \cdot 10^{-5}$ mol flask $^{-1}$ at t_2 ([Fig. 1](#)). During the same period, formate decreased by 61.1 %, acetate concentration increased by 14-fold and butyrate (not detected in vinasse) was produced ($5.30 \cdot 10^{-5}$ mol flask $^{-1}$ at t_2) ([Fig. 1](#)). At t_2 , no ethanol was detected, and isopropanol had totally disappeared from the solution ([Fig. 1](#)). At this time, CO_2 partial pressure equalled 0.057 atm ($1.28 \cdot 10^{-4}$ mol flask $^{-1}$) ([Fig. 1](#)), whereas neither N_2O nor CH_4 were detected (H_2 could not be detected at partial pressure lower than about 1 %). At t_2 , the I_{fm} to I_{rf} ratio equalled 67.0 % ([Table 4](#)), and total carbohydrate (I_{fc}) and 'polyols+carbohydrates' (I_{fc+p}) pools contributed to 41.4 % and 62.2 % of fermentation products (I_{rf}). Nitrate ($4.02 \cdot 10^{-5}$ mol flask $^{-1}$ initially) had totally disappeared at t_2 , whereas total NH_4^+ increased from $5.95 \cdot 10^{-6}$ to $9.17 \cdot 10^{-6}$ mol flask $^{-1}$ between t_0 and t_2 . Sulphate decreased of 7.8 % ($3.14 \cdot 10^{-5}$ mol flask $^{-1}$ at t_2). Between t_0 and t_2 , the minimum production of H_2 that should have been available for metal reduction, Q_{m-H_2} , equalled $1.23 \cdot 10^{-3}$ mol H_2 ([Table 4](#)), whereas the I_{aq-M} index, that considered only mobilised FeII and MnII corresponded to $6.91 \cdot 10^{-5}$ mol H_2 . The I_{aq-M} index represented then only 5.6 %

Figure 1: Evolution of organic compounds through incubation of V₂ and V₇ treatments.



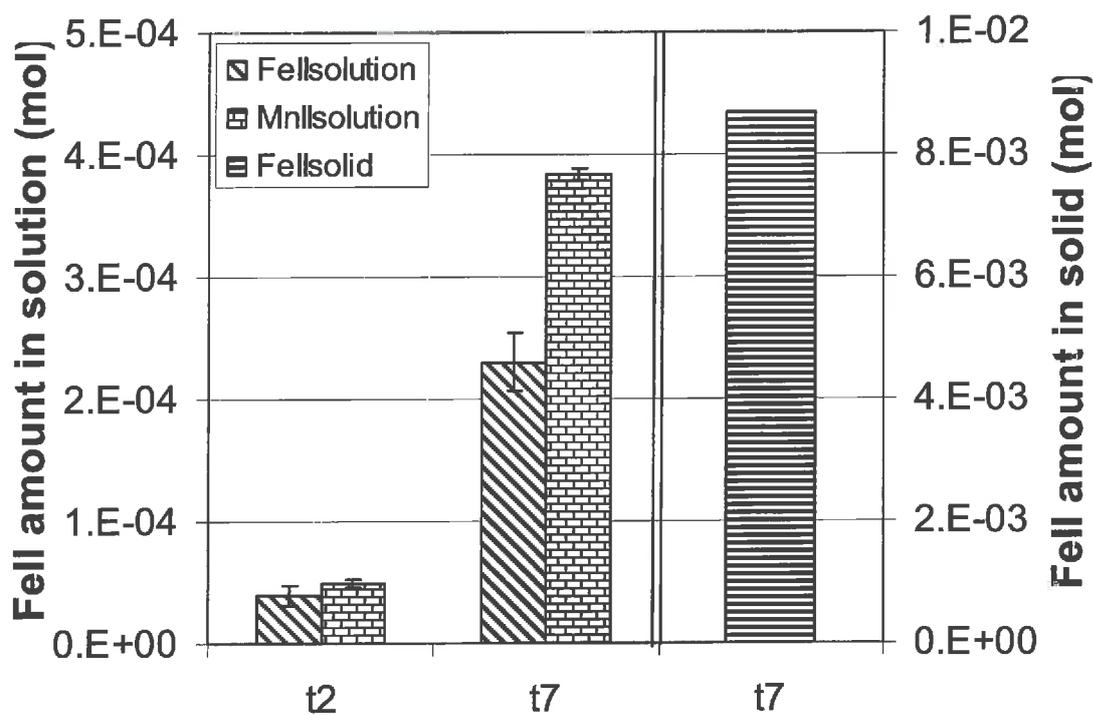
of Q_{m-H_2} (Table 4). Indeed at t_2 , $5.52 \cdot 10^{-4} \text{ mol l}^{-1}$ of FeII and $6.99 \cdot 10^{-4} \text{ mol l}^{-1}$ of MnII were in soil solution (Fig 2). At this date, speciation calculations indicated that 90.8 % of Fe were as free cations, and 7.5 and 1.7 % complexed with acetate and SO_4^{2-} , respectively. Similarly, 89.4 % of MnII were as free cations, whereas 7.7, 2.1 and 0.9 % were complexed with acetate SO_4^{2-} and CO_3^{2-} , respectively. Determination of log IAP (results not shown) indicated oversaturation of MnCO_3 , whereas saturation calculations suggested no formation of FeCO_3 , FeS, MnS, Fe(OH)_2 , Mn(OH)_2 and green rusts. pH increased from 5.31 to 5.45, whereas E_H decreased from 255.8 to 122.1 mV.

During the 7 days of the initial anaerobic incubation of the V_7 treatment, total carbohydrate, small sugars (C_5 , C_6 , C_{12}) and polyol concentrations decreased by 75.9, 99.0 and 96.1 %, respectively (Fig. 1). At t_7 , the small organic acids were consumed, except acetate, butyrate, formate, and propionate. Along the 7 d of anaerobiosis, propionate was produced to achieve $4.59 \cdot 10^{-4} \text{ mol flask}^{-1}$ at this date (Fig. 1). During the same period, formate decreased by 72.4 %, acetate concentration increased by 1.8-fold and butyrate (not detected in vinasse) was produced ($7.56 \cdot 10^{-4} \text{ mol flask}^{-1}$ at t_7) (Fig. 1). At t_7 , $3.98 \cdot 10^{-4} \text{ mol ethanol}$ was produced and isopropanol decreased by 98.7 %. At this date, CO_2 partial pressure equalled 0.358 atm ($8.01 \cdot 10^{-4} \text{ mol flask}^{-1}$), whereas neither N_2O nor CH_4 were detected, (H_2 could not be detected at partial pressure lower than about 1 %). At t_7 , the I_{fm} to I_{rf} ratio equalled 114.3 % (Table 3), and total carbohydrate (I_{fc}) and 'polyols+carbohydrates' (I_{fc+p}) pools contributed to 40.9 % and 61.6 % of fermentation products (I_{rf}). Nitrate ($4.13 \cdot 10^{-5} \text{ mol flask}^{-1}$ initially) had totally disappeared at t_7 , whereas total NH_4^+ in solution decreased from $7.24 \cdot 10^{-6}$ to $5.86 \cdot 10^{-6} \text{ mol flask}^{-1}$ between t_0 and t_7 . Sulphate decreased of 43.3 % ($6.72 \cdot 10^{-5} \text{ mol flask}^{-1}$ at t_7). Between t_0 and t_7 , the minimum production of H_2 that should have been available for metal reduction, Q_{m-H_2} , equalled $4.51 \cdot 10^{-3} \text{ mol H}_2$, whereas the I_{aq-M} index, that considered only mobilised FeII and MnII corresponded to $4.98 \cdot 10^{-4} \text{ mol H}_2$ (Table 4). The I_{aq-M} index represented then only 11.0 % of Q_{m-H_2} . Accounting for FeII in solids would have increased this index to 107 % of the Q_{m-H_2} (Table 4). Indeed at t_7 , $3.24 \cdot 10^{-3} \text{ mol l}^{-1}$ of FeII and $5.41 \cdot 10^{-3} \text{ mol l}^{-1}$ of MnII were in soil solution (Fig. 2), whereas 0.123 mol l^{-1} soil solution of FeII ($0.241 \text{ mol kg}^{-1}$ dry soil) were found in solid phases. The ratio of FeII in solution to FeII in solids phase was then equal approximately to 2.6 %. At this date, speciation calculations indicated that 76.1 % of Fe were as free cations, and 22.7 and 1.2 % complexed with acetate and SO_4^{2-} , respectively. Similarly, 68.6 % of MnII were as free cations, whereas 22.8, 1.3 and 8.6 % were complexed with acetate, SO_4^{2-} and CO_3^{2-} , respectively. Determination of log IAP

Table 4: Metabolic indexes for the period of V₂ and V₇ incubations.

| Indexes | units | T ₂ | T ₇ |
|---------------------------------|-----------------------------------|-----------------------|-----------------------|
| I_f | mol C | $2.16 \cdot 10^{-3}$ | $6.15 \cdot 10^{-3}$ |
| I_{rf} | mol C | $1.90 \cdot 10^{-3}$ | $7.06 \cdot 10^{-3}$ |
| I_{fm} | mol C | $1.27 \cdot 10^{-3}$ | $8.07 \cdot 10^{-3}$ |
| I_{fC} | mol C | $-5.24 \cdot 10^{-4}$ | $-2.89 \cdot 10^{-3}$ |
| I_{fC+p} | mol C | $-1.18 \cdot 10^{-3}$ | $-4.35 \cdot 10^{-3}$ |
| Q_{H_2} (mol H ₂) | mol H ₂ | $1.28 \cdot 10^{-3}$ | $4.76 \cdot 10^{-3}$ |
| $\Delta Q_{N-(NO_3^-+NO_2^-)}$ | mol N | $4.02 \cdot 10^{-5}$ | $4.13 \cdot 10^{-5}$ |
| $\Delta Q_{SO_4^{2-}}$ | mol SO ₄ ²⁻ | $2.66 \cdot 10^{-6}$ | $5.13 \cdot 10^{-5}$ |
| Q_{m-H_2} | mol H ₂ | $1.23 \cdot 10^{-3}$ | $4.51 \cdot 10^{-3}$ |
| $\Delta Q_{aq} \text{ FeII}$ | mol FeII | $3.91 \cdot 10^{-5}$ | $2.30 \cdot 10^{-4}$ |
| $\Delta Q_t \text{ FeII}$ | mol FeII | | $8.93 \cdot 10^{-3}$ |
| $\Delta Q_{aq} \text{ MnII}$ | mol MnII | $4.95 \cdot 10^{-5}$ | $3.84 \cdot 10^{-4}$ |

Figure 2: Reduced Fe in solution and solid phase for V₂ and V₇ treatments.



(results not shown) indicated an oversaturation of FeCO_3 , MnCO_3 , FeS whereas saturation calculations suggested no formation of MnS , $\text{Fe}(\text{OH})_2$ and $\text{Mn}(\text{OH})_2$ and green rusts. $p\text{H}$ increased from 5.10 to 5.80, whereas E_{H} decreased from 240.1 to -83.1 mV

IV.3.2 Microbial dynamics during the initial anaerobic incubations

From t_0 to t_2 , (or t_2') and from t_2' to t_7 , 'total' anaerobes increased. Similarly, the number densities of all the functional groups increased between t_0 and t_2 (or t_2') (Fig 3). In contrast, only FeIII reducers significantly grew between t_2' and t_7 . Number density of SO_4^{2-} reducers deviated significantly between treatments V_2 and V_7 after 2 d of incubation (i.e. at t_2 and t_2') (Fig 3); for fermentative bacteria, the deviation between t_2 and t_2' was nearly significant; other deviations between t_2 and t_2' were not significant. At last, the enumeration of 'total' anaerobes was higher than the sum of the considered functional communities (NO_3^- reducers, fermentative bacteria, FeIII reducers, and SO_4^{2-} reducers).

At t_2 of V_2 treatment, MPN enumerations corresponded to 3.0, 0.4 and 0.2 % of calculated number densities for N reducers, fermentative bacteria and SO_4^{2-} reducers, respectively. In contrast, FeIII reducer enumeration represented only 0.024 % of the calculated number density based on mobilised FeII only. At t_7 of V_7 treatment, MPN enumerations corresponded to 1.1, 0.1 and 0.02 % of biomass calculation for NO_3^- reducers, fermentative and SO_4^{2-} reducers, respectively. FeIII reducer enumeration represented only 0.007 % of the calculated number density based on the reduction of FeIII only. At t_2 , t_2' and t_7 , FeIII reducers were all SO_4^{2-} reducers (Table 5). The proportion of FeIII reducers that were also NO_3^- reducers was lower at t_7 (1.9 %) than at t_2 (8.4 %), whereas the proportion of FeIII reducers that had fermentative capacity did not vary (5.5-5.6 %) between t_2 and t_7 .

IV.3.3 Effect of addition of electron donor and acceptor to V_0 , V_2 and V_7 treatments

In order to check whether the initial anaerobic incubations increased the microbial ability of the soil slurry to reduce FeIII and MnIV and identify the effect of electron donors and acceptors on metal reductions, anaerobic incubations were prolonged for 2 more days for several flasks of treatments V_0 , V_2 and V_7 after renewing the atmosphere of the flasks and

Figure 3: Microbial functional communities dynamics through V₀, V₂ and V₇ incubations.

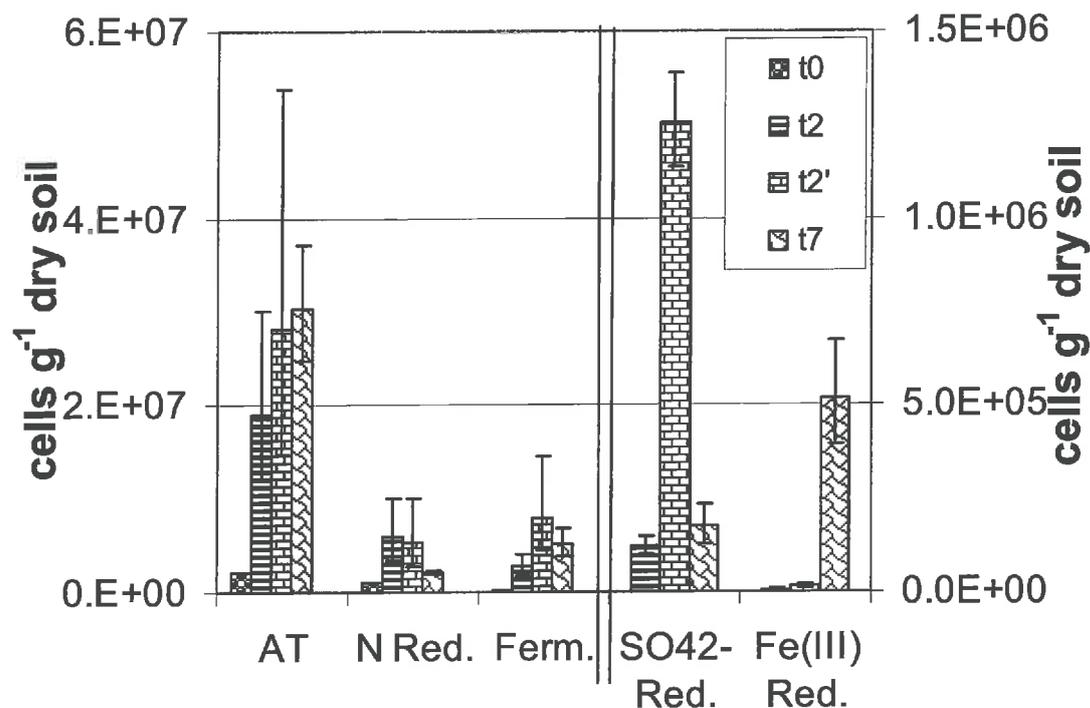


Table 5: Proportion of Fe reducer bacteria to reduce N oxides, SO₄²⁻ or to ferment.

| | MPN Fe(III) Reducers cells g ⁻¹ dry soil | Proportion of Fe(III) reducers able to | | |
|-------------------|---|---|-------------------|--|
| | | NO ₃ ⁻ reduction % | Fermentation % | SO ₄ ²⁻ reduction % |
| V ₀ | 1.35 10 ⁺¹ | - | - | - |
| V _{0-C} | 1.05 10 ⁺³ | 3.9 (2.7) | 1.5 (0.7) | 100.0 (0.0) |
| V _{0-L} | 6.56 10 ⁺⁴ | 7.1 (3.0) | 18.1 (5.8) | 100.0 (0.0) |
| V _{0-LN} | 1.31 10 ⁺⁴ | 0.4 (0.4) | 0.0 (0.0) | 100.0 (0.0) |
| V ₂ | 7.85 10 ⁺³ | 8.4 (2.8) | 5.6 (3.1) | 100.0 (0.0) |
| V _{2-C} | 8.90 10 ⁺⁴ | 5.8 (2.1) | 9.1 (3.7) | 100.0 (0.0) |
| V _{2-G} | 7.10 10 ⁺⁴ | 15.9 (12.6) | 17.1 (5.6) | 100.0 (0.0) |
| V _{2-H} | 2.01 10 ⁺⁵ | 7.7 (6.3) | 0.0 (0.0) | 100.0 (0.0) |
| V _{2-L} | 5.63 10 ⁺⁴ | 36.9 (13.5) | 0.1 (0.1) | 100.0 (0.0) |
| V _{2-LN} | 2.71 10 ⁺⁴ | 0.6 (0.5) | 0.0 (0.0) | 100.0 (0.0) |
| V _{2-LS} | 1.18 10 ⁺⁵ | 1.7 (1.3) | 0.0 (0.0) | 100.0 (0.0) |
| V ₇ | 5.16 10 ⁺⁵ | 1.9 (0.1) | 5.5 (1.8) | 100.0 (0.0) |
| V _{7-C} | 1.85 10 ⁺⁶ | 0.3 (0.1) | 2.2 (1.0) | 100.0 (0.0) |
| V _{7-L} | 8.77 10 ⁺⁵ | 0.4 (0.3) | 9.3 (3.3) | 100.0 (0.0) |
| V _{7-LN} | - | | | |

(X.X): error on the value

supplying the slurry with an electron donor and/or an electron acceptor. Major biotransformation types were scored (Table 5) using electron fluxes (cf section IV.2.3.2.).

Extended incubations of V_0 flasks with the addition of either milliQ water (V_{0-C}), lactate (V_{0-L}), or lactate and NO_3^- (V_{0-LN}) showed that (Table 5):

- NO_3^- disappeared in all treatments and no NO_2^- was detected at the end of these incubations. At t_2 , total NH_4^+ were equal to $1.78 \cdot 10^{-5}$, $2.98 \cdot 10^{-6}$ and $5.78 \cdot 10^{-6}$ mol.flask⁻¹ for V_{0-C} , V_{0-L} and V_{0-LN} , respectively;
- $[\text{SO}_4^{2-}]$ did not vary in the V_{0-C} treatment, whereas it increased by less than 10 % in the V_{0-L} treatment and was reduced to about 1 third in the V_{0-LN} treatment;
- no fermentation was observed in these 3 treatments;
- slight metal reductions was observed (Fig. 4).

pH of V_{0-C} , V_{0-L} and V_{0-LN} treatments were 5.75, 6.07 and 6.08 respectively.

At the end of V_{0-C} , V_{0-L} and V_{0-LN} treatments, FeIII reducers have significantly increased (Table 5). The addition of NO_3^- reduced significantly their growth in treatment V_{0-LN} in comparison with V_{0-L} treatment. At the end of V_{0-C} and V_{0-L} , V_{0-LN} treatments, the proportion of FeIII reducers having the ability to reduce the NO_3^- varied between 0.4 and 7.1 %, whereas the proportion of FeIII reducers that were able to ferment varied between 0 and 18.1 % (Table 5).

Extended incubations of V_2 flasks with the addition of either milliQ water (V_{2-C}), H_2 (V_{2-H}), lactate (V_{2-L}), glucose (V_{2-G}), lactate and NO_3^- (V_{2-LN}), or lactate and SO_4^{2-} (V_{2-LS}) showed that (Table 6):

- neither NO_3^- nor NO_2^- were detected at t_2 of V_2 incubation, except for the flasks supplied with lactate and NO_3^- (V_{2-LN}), in which all the NO_3^- initially present ($1.31 \cdot 10^{-3}$ mol flask⁻¹) was consumed while NO_2^- was produced ($9.11 \cdot 10^{-5}$ mol flask⁻¹ at the end of the incubation). At this time, total NH_4^+ in V_{2-LN} was 8 – 45 higher ($7.57 \cdot 10^{-5}$ mol flask⁻¹) than other V_2 treatment after compound addition;
- fermentations occurred in all treatments. No H_2 was detected at the end of all additional treatments. At t_2 of V_2 incubation, there were still carbohydrates ($9.85 \cdot 10^{-5}$ mol), from which about 2 thirds were consumed thereafter in treatments V_{2-C} , V_{2-L} and V_{2-H} ., whereas the addition of glucose ($2.74 \cdot 10^{-4}$ mol flask⁻¹) in treatment V_{2-G} led to the consumption of 50 % of total carbohydrates (all added glucose was consumed);
- between 1 half and 2 thirds of the remaining SO_4^{2-} were consumed in treatments V_{2-C} , V_{2-G} , V_{2-H} , whereas about 1 tenth disappeared in treatment V_{2-LN} . In V_{2-LS} treatment ($8.17 \cdot 10^{-4}$ mol of added SO_4^{2-} flask⁻¹), there was no significant trend in SO_4^{2-}

Table 6: Classification of activity according to electron fluxes.

| TREATMENT | METABOLISMS | | | | |
|-------------------|--|--------------|---|----------------------------|----------------|
| | NO ₃ ⁻ reduction | Fermentation | SO ₄ ²⁻ reduction | FeIII reduction (solution) | MnIV reduction |
| V _{0-C} | ↗↗↗ | ↘ | ↘↘ | ↗ | 0 |
| V _{0-L} | ↗↗↗ | ↘ | ↘↘ | 0 | ↗ |
| V _{0-LN} | ↗↗↗↗ | ↘ | ↗↗↗ | 0 | ↗ |
| V _{2-C} | 0 | ↗↗↗ | ↗↗↗ | ↘↘ | ↗↗ |
| V _{2-G} | 0 | ↗↗↗↗ | ↗↗↗ | ↗↗ | ↗↗↗ |
| V _{2-H} | 0 | ↗↗↗ | ↗↗↗ | ↘↘ | ↗↗ |
| V _{2-L} | 0 | ↗↗↗ | ↗↗↗ | ↘↘ | ↗↗ |
| V _{2-LN} | ↗↗↗↗ | ↗↗↗ | ↗↗ | ↘↘ | ↘↘ |
| V _{2-LS} | 0 | ↗↗↗ | -* | ↘↘ | ↗↗ |
| V _{7-C} | 0 | ↘↘↘ | ↗↗↗ | ↗↗ | ↗↗ |
| V _{7-L} | 0 | ↘↘↘ | ↗↗↗ | ↗ | ↗ |
| V _{7-LN} | ↗↗↗↗ | ↘↘↘ | ↘↘↘ | ↘↘↘ | ↘↘↘ |

* SO₄²⁻ deviation was in the error scale limits

Activity notations were assigned as increase arrows for reduction and reverse arrows for oxidations using following lognormal scale of electron flux as boundaries:

0 (10⁻⁷ - 10⁻⁶ mol e⁻), ↗ or ↘ (10⁻⁶ - 10⁻⁵ mol e⁻), ↗↗ or ↘↘ (10⁻⁵ - 10⁻⁴ mol e⁻), ↗↗↗ or ↘↘↘ (10⁻⁴ - 10⁻³ mol e⁻), ↗↗↗↗ or ↘↘↘↘ (10⁻³ - 10⁻² mol e⁻)

concentration over the additional 2 d of incubation. All lactate was consumed in V_{2-G}, V_{2-L}, V_{2-LN} and V_{2-LS}. In V_{2-C} and V_{2-H} treatment, lactate increased by 2-fold compared to t₂ value.

- metal mobilisations varied with (i) the element (Fe or Mn) and the treatment (Fig. 4), and considering only their mobilised amounts could bias interpretations on representation of metal reduction. At the end of all second incubations, except for V_{2-G} and V_{2-LN}, mobilised FeII decreased of 55 – 80 % in comparison to date t₂ (Fig. 4). In contrast, mobilised MnII concentration increased (1.3-1.6 fold) for all treatments with the exception of V_{2-G} and V_{2-LN}. Addition of glucose led to a 2.5-fold increase of mobilised FeII. Mössbauer analysis resulted to incorrect data due to microbial activities which carried out after sampling and before analysis for V_{2-C} and V_{2-L} treatments. Addition of lactate and NO₃⁻ (V_{2-LN}) led to a decrease of 98.2 and 55 % of mobilised FeII and MnII, respectively. Despite uncertainties on Mössbauer analyses, addition of NO₃⁻ in V_{2-LN} treatment resulted unquestionably to nearly complete disappearance of FeII in solid phase.

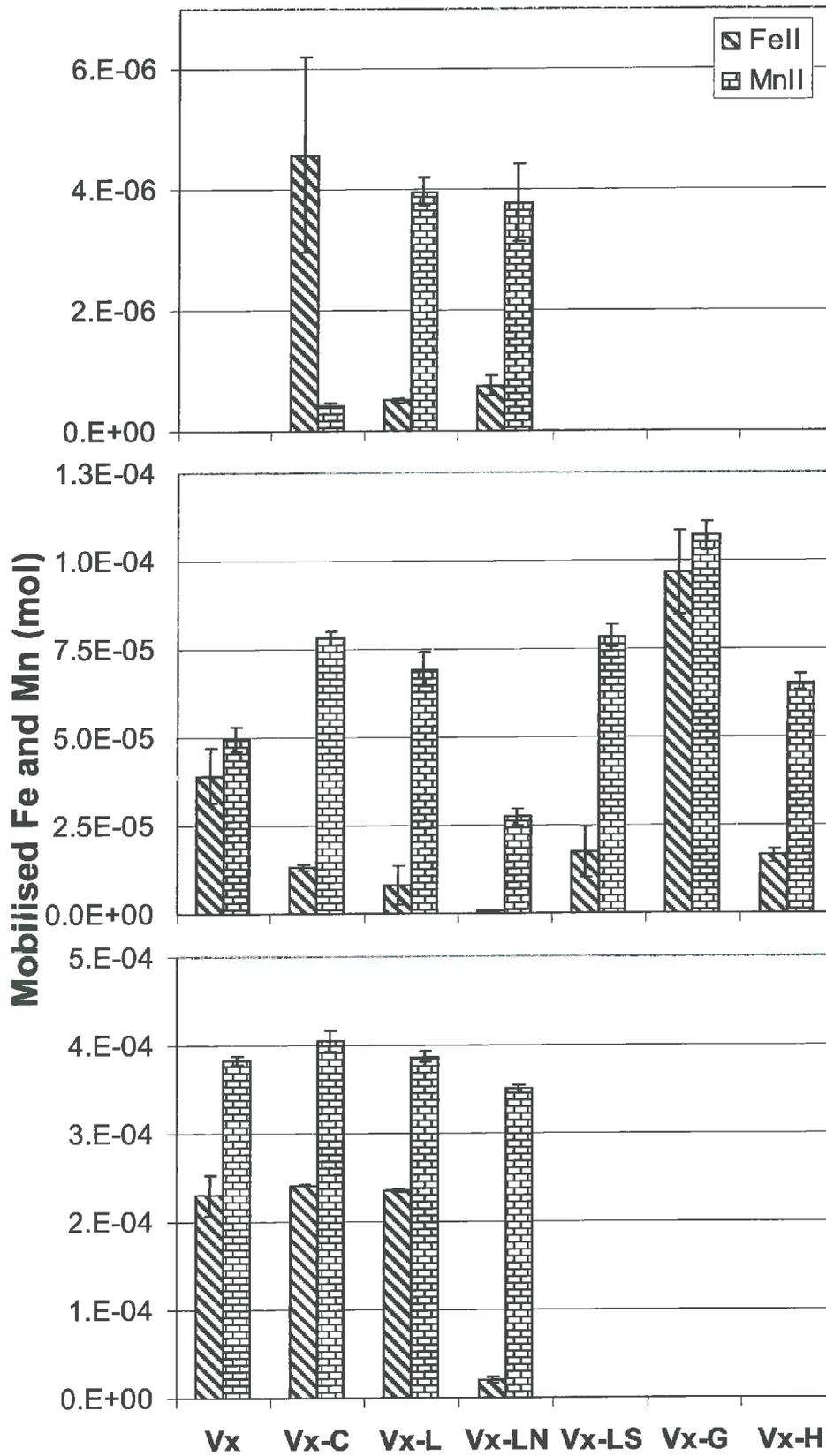
pH of V_{2-C}, V_{2-G}, V_{2-H}, V_{2-L}, V_{2-LN} and V_{2-LS} treatments were 5.51, 5.10, 5.97, 5.92, 6.56 and 6.07.

At the end of V_{2-C}, V_{2-G}, V_{2-H}, V_{2-L} treatments, FeIII reducers had significantly increased in comparison to enumeration at t₂ of V₂ treatment (Table 5). However, added electron donors did not have significant effect on growth of FeIII reducers in comparison to V_{2-C}, with the exception of H₂. The addition of SO₄²⁻ did not enhance significantly the FeIII reducers, whereas NO₃⁻ addition led to 50 % cell decrease compared to enumeration performed in V_{2-L} treatment. In V_{2-G}, 15.9 % of the FeIII reducers were also NO₃⁻-reducers, and 17.1 % were able to ferment. In V_{2-L}, 36.9 % of the FeIII reducers could denitrify. In other V₂ treatments, about 0.6 – 7.7 % of FeIII reducers could denitrify, whereas nearly none was able to ferment, except in V_{2-C} and V_{2-G} with 9.1 and 17.1 % of FeIII reducers which could ferment.

Extended incubations of V₇ slurry flasks with the addition of either only MilliQ water (V_{7-C}), lactate (V_{7-L}), or lactate and NO₃⁻ (V_{7-LN}) showed that (Table 6):

- neither NO₃⁻ nor NO₂⁻ were detected at t₇ of V₇ incubation, except for the flasks supplied with lactate and NO₃⁻ (V_{7-LN}), in which all the NO₃⁻ (1.31 10⁻³ mol) was consumed, while NO₂⁻ was produced (3.06 10⁻⁴ mol at the end of the incubation). At this time, total NH₄⁺ in V_{7-C} and V_{7-LN} were as 1.7 and 1.3- fold greater than t₇ value. Total NH₄⁺ in V_{7-L} decreased of 34 %;

Figure 4: Mobilised Fe(II) after addition of donors or/and acceptors of electrons.



- fermentative metabolism was based on total carbohydrates consumption. In all t_{7-C} , t_{7-L} and t_{7-LN} treatments, total carbohydrates increased significantly by 15.5, 64.6 and 82.2 % of t_7 value, respectively;
- remaining SO_4^{2-} decreased by 51 and 58 % in V_{7-C} and V_{7-L} treatments, whereas $[SO_4^{2-}]$ increased of 20 % at the end of V_{7-LN} treatment;
- metal reductions in solution slightly increased by 5 – 6 % for FeII and 0 - 2 % for MnII at the end of V_{7-C} and V_{7-L} treatments. Addition of NO_3^- (V_{7-LN}) led to a decrease by 91 % of FeII_(aq) and 9 % of MnII_(aq). Mössbauer analyse for the 3 treatments will be performed again to verify consistency of FeII evolution in solution and in solid phase.

pH at the end of V_{7-C} , V_{7-L} and V_{7-LN} treatments, were 5.94, 5.01 and 5.98, respectively.

At the end of V_{7-C} and V_{7-L} treatments, FeIII reducers had not changed significantly (Table 5). However no FeIII reducing bacteria were observed after addition of lactate and NO_3^- at the end of V_{7-LN} treatment. At the end of V_{7-C} and V_{7-L} treatments, the proportion of FeIII reducers having the capacity to reduce NO_3^- was about 0.3 – 0.4 % whereas the proportion of FeIII reducers that were able to ferment varied between 2.2 and 9.3 %.

IV.4 Discussion and Conclusions

IV.4.1 Biogeochemical evolution over the 2 incubation periods

Fermentations and true acetogenic transformations of butyrate and ethanol explained most of initial organic C biotransformations, as it can be seen from the ratio of I_{fm} to I_{rf} indexes (67.0 and 114.3 % at t_2 for V_2 and t_7 for V_7 , respectively), and from the ratio of I_{FC+p} to I_{rf} (62.2 and 61.6 % at t_2 for V_2 and t_7 for V_7 , respectively) (Table 4). Pautremat et al. (Section III) who performed batch incubation at 25°C of the same soil with the same vinasse at 1:10 dilution, obtained lower I_{fm}/I_{rf} ratio after 2 and 7 d of anaerobiosis (57 and 56 %, respectively), whereas I_{FC+p}/I_{rf} were higher after identical period of anaerobiosis (86.6 and 82.6 % after 2 and 7). I_{fm} to I_{rf} ratio lower than 1 (V_2) suggested that formate was only partly dissociated or oxidised, as long as it tends to 1 for longer times (Section III); whereas I_{fm} to I_{rf} ratio higher than 1 (V_7) suggested that either non fermentative processes are also involved in the mineralization of organic matter or acetate is used mineralised or used for CH_4 production. However no CH_4 emission was detected after 7 d of anaerobiosis, according to previous

experiments (Section III). The $I_{\text{FC+p}}$ to I_{rf} in V_2 indicated that compounds other than carbohydrates, polyols, and other fatty acids participated to the production of acetate, butyrate and ethanol. This is the case of proteins that represented 2.5 % of organic C supplied with the vinasse. The $I_{\text{FC+p}}$ to I_{rf} lower than 1 also suggested that organic compounds did not directly participate to FeIII and MnIV reduction, and led us to assume that the reduction of FeIII and MnIV was mainly coupled with H_2 oxidation.

We estimated that $1.28 \cdot 10^{-3}$ and $4.76 \cdot 10^{-3}$ mol H_2 (Q_{H_2}) could have been produced during initial V_2 and V_7 incubations through formate lysis and true acetogenesis. Initial NO_3^- reduction into NO_2^- that could oxidise formate was negligible due to initial NO_3^- content. SO_4^{2-} reduction that could either oxidise H_2 or explain a lack of H_2 production represented less than 0.8 % and 4.3 % of the estimated H_2 gross production for V_2 and V_7 , respectively. In addition, no CH_4 production that could consume H_2 was detected during these short periods. Therefore neglecting also homoacetogenesis since the I_{fm} to I_{rf} ratio tend to values about 1 for longer time (21 d) with the same soil and vinasse (Section III), minimum H_2 available for metal reduction, $Q_{\text{m-H}_2}$, was equal to $4.51 \cdot 10^{-3}$ mol H_2 in V_7 incubation. Experimental ratio of FeII in solution versus FeII in solid was 2.7 % at t_7 for V_7 treatment. As mentioned already (Dassonville, 2003; Ona-Nguéma, 2003), we concluded that only a few percent of reduced FeII were in solution. The amount of produced FeII in solids could have consumed 99 % of $Q_{\text{m-H}_2}$ mol of H_2 at the end of V_7 incubation. Since FeII was mainly in solids, when considering FeII in solution and in solids, and MnII, the $I_{\text{S-M}}$ index extended to FeII in solids represented 107 % of $Q_{\text{m-H}_2}$ mol of H_2 in V_7 treatment. Considering identical ratio for MnII in solution versus MnII in solids and FeII in solution versus FeII in solids, the I_{SM} index extended to FeII and MnII in solids would have, represented 429 % at t_7 .

In previous studies, reduced FeII and MnII were mainly detected in solids as green rusts, precursors of magnetite, vivianite, carbonates and sulfides (Dong et al., 2000; Dong et al., 2003; Glasauer et al., 2003), and analyzing only the soil solution would have led to large underestimation of these reductions. As similar in our experiments, the mobilised FeII corresponded to about 2.7 % of reduced FeII for V_7 incubation. This could partly result from precipitations of minerals such as FeCO_3 and FeS since the solution was oversaturated for these ones at t_7 for V_7 , and partly from other processes including (i) a reduction of FeIII without any mobilisation, and (ii) the adsorption of FeII on solids including FeIII oxyhydroxides and its evolution as siderite in presence of H_2CO_3 , vivianite in presence of

phosphate ions, and magnetite or green rust according to FeII production kinetic (Ona-Nguéma, 2003).

During the second incubation period of the V₂ treatment, FeII_(aq) slightly decreased in V_{2-C} and V_{2-L}. The change of the atmosphere between the 1st and the 2nd incubation periods led to a sudden fall in CO₂ partial pressure from 5.72 10⁻² atm to nearly 0 atm; this change increases the pH of the solution and can reduce the mobilisation of FeII: the pH of the slurry equalled 5.44 at the end of the first incubation period and was equal to 5.51, 5.92 and 6.56 at the end of the second incubation period for V_{2-C}, V_{2-L} and V_{2-LN}, respectively. However, FeII disappeared simultaneously in solution (Fig. 4) and solids in V_{2-LN} treatment; in this last case, we may hypothesise that NO₃⁻ addition had simultaneously a toxic effect through the accumulation of NO₂⁻ (1.29 10⁻³ mol l⁻¹ at the end of V_{2-LN}) and could have also induced abiotic oxidation of FeII through the reduction of NO₃⁻ (Murase and Kimura, 1997) as it has already been noted for FeII in green rusts (Hansen et al., 1996) or abiotic oxidation of FeII to FeIII by NO₂⁻ (Komatsu et al., 1978). The additions of water, glucose or SO₄²⁻ enhanced the mobilisation of MnII, whereas addition of H₂ or NO₃⁻ induced a decrease in this mobilisation. NO₃⁻ affected less the fate of MnII than FeII.

During the second period of V₇ incubation, mobilised FeII disappeared in V_{7-LN}, and slightly decreased in V_{7-C} and V_{7-L}. As discussed for additional V₂ treatments, the switch to N₂ atmosphere at the end of 1st incubation period led to a fall in of CO₂ partial pressure from 0.36 atm to nearly 0 atm, an increase the pH of the slurry solution (the pH of the slurry equalled 4.48 at the end of the first incubation period and was equal to 5.93, 5.01 and 5.98 at the end of V_{7-C}, V_{7-L} and V_{7-LN} incubations, respectively), and therefore a slight decrease of FeII_(aq) concentrations. As observed after addition of NO₃⁻ in V₂, FeII decreased simultaneously in solution (Fig. 4) and solids in V_{7-LN} treatment. As for V_{2-LN}, these observations could be attributed to the toxic effect of accumulated NO₂⁻ (4.32 10⁻³ mol.l⁻¹ at the end of V_{7-LN}) and abiotic oxidation of FeII through the reduction of NO₃⁻ (Murase and Kimura, 1997) or reduction of NO₂⁻ (Komatsu et al., 1978). Addition of NO₃⁻ slightly inhibited MnII mobilisation, whereas addition of water and lactate had no significant effect on MnII mobilisation.

During the "second" incubation period of the V₀ treatment, FeII in solution increased in all treatments even if only a small decrease was observed in V_{0-LN} in comparison to V_{0-L}. In absence of NO₂⁻ at the end of incubation, NO₃⁻ reduction coupled to FeII abiotic oxidation may have slightly contributed to reduction of mobilised FeII in this treatment (Fig. 4).

Addition of lactate and lactate and NO_3^- favoured MnII mobilisation. The pH of the slurry equalled 5.48 at the beginning of the incubation period and was equal to 5.75, 6.07 and 6.07 at the end of the second incubation period for V_{0-C} , V_{0-L} and V_{0-LN} , respectively.

IV.4.2 Relationship between FeIII reduction and the dynamic of FeIII reducers

Bacteria functional groups did not show linear or exponential dynamics, with the exception of FeIII reducers and 'total' anaerobes (Fig. 3). Organic matter may have been a limiting factor of growth between t_2' and t_7 , for fermentative bacteria and SO_4^{2-} reducers, whereas NO_3^- may have been the limiting factor for NO_3^- reducers. This hypothesis was sustained (i) on one hand by the variations in NO_3^- , carbohydrates and substrates easily consumed by SO_4^{2-} reducers (e.g. lactate), (ii) on the other hand by the ratio of increases in enumerated cells versus calculated number of cells: these ratio decreased between V_2 and V_7 treatments from 3.0 to 1.1, 0.4 to 0.1 and 0.2 to 0.02 % for NO_3^- reducers, fermentative bacteria and SO_4^{2-} reducers, respectively. Indeed, cells enumeration enables only to indirectly access to a net increase in cultivable bacteria, which accounts for growth and mortality. For example, after NO_3^- disappearance, NO_3^- reducers that can not use other catabolic pathway should decrease due to substrate limitation (Achnich et al., 1995).

As in Pautremat et al (Section III), fermentations and true acetogenesis were the main microbial process, favoured by vinasse inputs. However it was then surprising that fermenting-bacteria enumeration was low with regard to the enumeration of 'total' anaerobes. We assume that the medium of culture must have been too selective to the fermenting bacteria. Then sum of functional groups could not be compared to 'total' anaerobes.

The growth of FeIII reducers was concomitant to an increase in FeII at t_2 and t_7 . However the increase in MPN enumeration (Fig 3) from t_0 to t_7 was low with regard to the calculated increase in bacteria number (0.0073 %), all the more several FeIII reducers can reduce MnIV (Ghiorse, 1988) and accounting for MnIV reduced would have decreased these ratios. This suggested that either one or some of the following hypothesis:

- low MPN enumeration were attributed to too selective medium and/or the low cultivability of FeIII reducers. However, FeIII citrate as electron acceptor in the medium

should induce an overestimation of FeIII-reducers, due to its availability, which is not representative of reduction on solid phases in soil.

- an important proportion of FeII was reduced by abiotic reactions, such as abiotic reoxidation of HS^- to S^0 or SO_4^{2-} concomitant to FeIII reduction to FeII (Murase and Kimura, 1997);
- bacteria of other functional communities could reduce metal with a low or no gaining energy on the reaction (Comm. A. Richaume), and therefore could not develop on culture medium for FeIII reducers. For example, denitrifiers were identified as able to reduce FeIII without gaining in energy.

After the initial incubation, the addition of substrates was performed to assess their effect on FeIII reduction and FeIII reducer dynamics. The lack of systematic positive correlation between the growth of FeIII reducers and FeIII reduction confirms the indirect contribution of others communities to FeIII reduction. During the 2 additional days of incubations of V_{x-C} treatments (i.e. after the addition of water), the density number of FeIII reducers and the amount of reduced FeIII increased, whatever the duration of the initial anaerobic incubation. During the 2 additional days of incubations of V_{x-L} treatments (i.e. after the addition of lactate) FeIII reduction was enhanced, whereas no beneficial effect on growth was observed in comparison to V_{x-C} treatments, at the exception of V_{0-L} treatment where organic matter was more limiting. Glucose addition had a positive effect on FeIII mobilisation that was not correlated to an increase in FeIII reducers. In contrast, H_2 addition enhanced greatly FeIII reducers growth, whereas it has little effect on mobilised FeII. This suggested that H_2 addition stimulated the growth of FeIII reducers as in paddy soil slurries (Achnich et al., 1995), and like Lovley et al. (1989) and Lovley and Goodwin (1988) considered H_2 as the preferential electron donors for microbial FeIII reduction, although small sugars and fatty acids can also be used by FeIII reducers as electron donors. In contrast, lactate and glucose after fermentation could enhance SO_4^{2-} reduction that could increase the subsequent abiotic reduction of FeIII. Addition of SO_4^{2-} had a slight positive effect on growth of FeIII reducers and on FeIII mobilisation that could be due to its indirect contribution to FeIII reduction although SO_4^{2-} concentration was probably not limiting for SO_4^{2-} reduction. On the opposite, NO_3^- addition had a negative effect on FeIII reduction and growth of FeIII reducers in V_{2-LN} and V_{7-LN} treatments; it led to the complete disappearance of FeIII reducers in V_{7-LN} treatment. The mortality could be attributed to NO_2^- toxicity, especially in V_{7-LN} , whereas

the oxidation of FeII in solids could be coupled to the abiotic reduction of NO_3^- into NH_4^+ as it was already observed by Hansen et al. (1996) for FeII in green rust. In V_{2-LN} and V_{7-LN} treatments, NO_3^- was completely consumed, whereas NO_2^- accumulated in soil to concentrations upper to microbial toxicity levels ($3.02 \cdot 10^{-4} \text{ g N g}^{-1}$ dry soil (V_{2-LN}); $1.64 \cdot 10^{-3} \text{ g N g}^{-1}$ dry soil (V_{7-LN})) (Bancroft et al., 1978). Wodzinski et al. (1978) supported the view of a pH dependency of NO_2^- toxicity, with toxicity concentrations of $1 \cdot 10^{-4} \text{ g N-NO}_2^- \text{ g}^{-1}$ soil at pH 6 and of $1 \cdot 10^{-3} \text{ g N-NO}_2^- \text{ g}^{-1}$ soil at pH 7.7. pH was equal to 6.56 and 5.98 at the end of V_{2-LN} and V_{7-LN} incubations, respectively, which could explain that NO_2^- had a greater toxic effect in V_{7-LN} .

For all the treatments and dates, FeIII reducers could reduce the SO_4^{2-} , whereas only a few of them were able to reduce NO_3^- or ferment. At t_2 and t_7 , less than 10 % of them were also NO_3^- reducers or fermentative bacteria (Table 5). The functional evolutions between dates t_2 and t_7 were coherent with the expression of these functions. After addition of substrates, the proportion of FeIII reducers combining fermentative and NO_3^- reducing capabilities changed with the supplied substrates. As observed for FeIII reducer enumerations, the addition of electron acceptors had a depressive effect on the capacity of FeIII reducers to denitrify or ferment. Lactate addition stimulated the capacity of FeIII reducers to ferment in V_{0-L} and in V_{7-L} . No identical effect was observed in V_{2-L} . At the end of V_2 incubation, lactate was still in solution, whereas in V_{0-L} and V_{7-L} , where it was not detected. Lactate addition appeared then as a new source of electron. Glucose addition stimulated the capacity of FeIII reducers to ferment and to reduce N oxides. In contrary it seemed that added H_2 inhibited the fermenting-capacity of FeIII reducers. This observation may be due to the replacement of the flask atmosphere at t_2 by 100 % of H_2 , which may have inhibiting fermentation. Accordingly, total carbohydrates consumption after H_2 addition was smaller than this in V_{2-C} (results not shown).

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V Devenir du FeII et du MnII lors d'un passage de l'anaérobiose à l'aérobiose

L'anaérobiose est un phénomène transitoire dans l'espace et le temps. Afin d'évaluer les risques de transfert de métaux vers les eaux de surface ou les aquifères, il est nécessaire de prendre en compte leurs évolutions lors d'un retour à l'aérobiose. Les alternances entre anaérobiose et aérobiose soulèvent des interrogations relatives (i) aux effets dans un système sol sur la minéralogie, géochimie et la biodiversité et de la fonctionnalité de la microflore, (ii) à l'impact de la coexistence de composés réduits et oxydés. A notre connaissance, les transformations biogéochimiques et la résilience ou non d'un système après alternances restent peu étudiées. Ce travail avait ainsi pour objectifs de caractériser au cours d'un épisode aérobie faisant suite à une incubation en anaérobiose (i) le devenir des formes réduites de Fe et Mn en phases liquide et solide (ii) l'évolution des composés organiques et minéraux, notamment ceux pouvant complexer les métaux, et (iii) les changements de biodiversité microbienne.

Les expériences étaient basées sur des incubations initiales en batch et en anaérobiose d'un Ferralsol additionné de vinasse de rhumerie pendant 2, 7 ou 21 j, suivies de 2 j d'incubation en aérobiose. Les analyses incluaient des suivis (i) des concentrations du FeII et MnII en solution, du FeII en phase solide, (ii) des concentrations en solutés minéraux autres et organiques et (iii) des suivis de biodiversité microbienne du sol au travers de la PCR-SSCP.

Lors du retour à l'aérobiose, les FeII et MnII en solution ont présenté des évolutions contrastées avec une disparition du FeII très rapide et sur une durée corrélée à la durée de la phase initiale anaérobie, alors que MnII se maintenait en solution sans évolution apparente. De même, les évolutions de solutés organiques ont été faibles au cours de la phase aérobiose et suggèrent de faibles activités microbiennes aérobies sans doute dues aux effets néfastes de l'anaérobiose sur certains micro-organismes aérobies. Les données de PCR-SSCP ont montré que (i) la phase anaérobie aurait favorisé la microflore anaérobie et affaibli les aérobies, mais (ii) qu'après 2 j d'aérobiose, les micro-organismes de l'ensemble des traitements semblent converger vers une biodiversité de profil aérobie, sans pouvoir parler d'un retour à l'état initial, donc d'une résilience du système.

Ce chapitre 5 correspond à un article qui sera soumis à la revue *Geochimica et Cosmochimica Acta* sous les titre " Fate of reduced Fe and Mn in a soil amended with a vinasse and incubated anaerobically after a return to aerobic conditions" avec pour auteurs : N. Pautremat, P. Renault, P. Cazevielle, J.C. Jumas, M. Womes, J.J. Godon and S. Mourey.

Fate of reduced Fe and Mn in a soil amended with a vinasse and incubated anaerobically after a return to aerobic conditions.

Abstract

Spreading liquid organic waste on soil enhances the risk of transitory anaerobic phase and metal mobilisations. This work aimed to characterise during aerobic incubations that follow initial anaerobic periods (i) the fates of reduced Fe and Mn in liquid and solid phases, (ii) the fates of mineral and organic small compounds that complex these metals, and (iii) biodiversity changes. A Ferralsol (FAO classification) was first supplied with a rum vinasse, then incubated during either 2, 7 or 21 d under anaerobic conditions, and finally incubated under aerobic conditions for 2 d. Analyses included (i) FeII and MnII concentrations in solution, as well as FeII to FeIII ratio in solids, (ii) concentrations of organic and mineral ligands, and (iii) microbial biodiversity. In addition, a model accounting for FeII mobilisation and oxidation was proposed and fitted to experimental data. During the initial aerobic incubation, the reduction and mobilisation of FeII and MnII increased with time. At 21 d, FeII in solids included paramagnetic FeII like green rusts that nearly totally disappeared during the 2 d of aerobiosis, paramagnetic FeII minerals like sulfides that did not evolve during the aerobic period, and magnetic FeII/FeIII that partly disappeared during the aerobic period. During the subsequent aerobic phases, FeII immobilisation was complete after few hours and suggested an abiotic oxidation in solution. The existence of FeII in solids and its partial dissolution after 7 and 21 d increased the time required for mobilised FeII to disappear from the solution, as it was confirmed by fitting simulated data to experimental ones. In contrast to FeII oxidation, abiotic oxidation of MnII was too low to be observed into 2 d of aerobiosis, and biologic MnII oxidation did not proceed and is probably linked to low microbial activities after the return to aerobiosis. We suggested combined effect initial anaerobiosis and MnII toxicities on aerobic microorganisms. PCR-SSCP indicated the dominance of few distinct populations after the anaerobic periods, and later changes of PCR-SSCP profiles without returning to the initial SSCP profiles.

V.1 Introduction

Increasing the production of liquid wastes had led modern societies to consider recycling opportunities (Friedel et al, 2000). Beside positive effects (e.g. on soil structure (Angin et al., 2005) and plant yield (Gemtos et al., 1999)), their spreading on the soil can have adverse environmental and agricultural impacts (Speir et al., 2003). In particular, the concomitant supply of water and organic compounds enhances the risk of anaerobiosis (Chen et al., 2003) and favours the reduction and mobilisation of Fe and Mn (Quantin et al., 2001). On the one hand, the fates of Fe and Mn affect others metal fates (U, Co, Pb, Ni, Cu, Zn) (Duff et al., 2002). On the other hand, Fe and Mn are micronutrients that become toxic beyond concentrations, which vary according to the element and the organisms (Hue et al., 2001; Seaker, 1991; Rajapaksha et al., 2004). Although these concentrations can be reached in anaerobic conditions (Quantin et al., 2002), such conditions are generally limited in space and time (Brune et al., 2000; Roden et al., 2004). Fluctuations between anoxia and aerated conditions or spatial juxtapositions of anaerobic/aerobic sites are more often observed in natural environments (Weiss et al., 2003, Lüdemann et al., 2000; Oguz et al., 2001).

Anaerobiosis favours the reduction and mobilisation of Fe and Mn (Straub, 2001), as well as the production and accumulation of mineral compounds (HCO_3^- (Kimura, 2000), NH_3 (Tiedje et al., 1984), HS^- (Legall et Fauque, 1988)) and organic ones (fatty acids (Urrutia et al., 1988) and amino acids (Li and Byrne, 1990)) that can complex these metals and thus enhance their mobilisation. Most of FeII and MnII in soils supplied with vinasse and incubated anaerobically may be in solids (Dassonville, 2003). The return to aerobic conditions induces the oxidation of these metals (Frankel and Bazylinski, 2003), the exit of CO_2 , and the oxidation of mineral and organic ligands (McGuire et al., 2001; van Bodegom et al., 2001). The oxidations of Fe and Mn may result from microbial and abiotic processes (Morgan, 2005; Tebo et He, 1999). More precisely, the abiotic oxidation of MnII is generally a slow process with regard to microbial ones (Morgan, 2005). Then in well aerated environment medium, MnII would be often biologically oxidized. In contrast under oxic conditions at circumneutral pH, FeII oxidation is a rapid and spontaneous abiotic reaction (Stumm et Morgan, 1996), and microbial oxidation of FeII under these conditions can then be considered as a minor process (Emerson and Weiss, 2004). Several environmental factors affect these oxidations:

- Firstly, the complexations of FeII and MnII have been shown to reduce the FeII and MnII oxidation rates (Millero et al, 1987);
- Secondly, the pH of the soil solution also affects the oxidations of FeII and MnII. In particular, abiotic oxidation of FeII was 100 times greater with a pH increase of 1 unit (Tebo and He, 1999) for pH higher than 5. In contrast, abiotic oxidation of MnII has been observed only for pH higher than 8 (Morgan, 2005), the abiotic oxidation of MnII decreasing 70 times with a pH decrease of 1 unit for pH higher than 8. MnII oxidizing bacteria are neutrophilic ones (Tebo and He, 1999) that belong to various genera, such as *Leptothrix*, *Crenothrix*, *Hypomicrobium*, *Siderocapsa* and *Metallogenium* (Tebo et He, 1999);
- Thirdly, Mn and Fe oxides as well as bacteria walls can also catalyse the oxidation of FeII and MnII, and increase abiotic oxidation rate of 20 times in the case of FeII (Morgan and Davies, 1989).

Thus, Morgan (2005) estimated half life of MnII oxidation in solution at pH 8 about 10 h when performed by bacteria, 30 d when catalyzed by Fe or Mn oxides, and 400 d for abiotic oxidation in solution without catalyst. Similarly, Morgan and Davies (1989) noted that the rate of abiotic oxidation of FeII at circumneutral pH was increased 20 times after the addition of goethite and ferrihydrite. However, the oxidation of FeII and MnII and their precipitation as FeIII oxy-hydroxides (ferrihydrite, hematite, goethite, lepidocrocite etc.) and MnIII/IV (bixbyite, birnessite, pyrolusite, nsutite etc.) (Tebo and He, 1999) will also induce the dissolution of FeII and MnII from various solids, including carbonates (Duckworth and Martin, 2004), sulphides (Neal et al., 2001) and oxyhydroxides (Tebo and He, 1999). To our knowledge, only a few studies deal with soil geochemical resiliency after an anaerobic temporary event (mineralogy, C.E.C.) (Favre et al., 2002). The fates of Fe and Mn also depends on the fates of mineral and organic ligands, as well as the alternation between anaerobic and aerobic activities. Several studies deal with the oxidation of various ligands including NH_4^+ (van Bodegom et al., 2001), and sulfides (Edwards et al., 2000). However, these oxidations depend partly (sulfides) or totally (NH_4^+) from microbial processes. To our knowledge, only a few studies deal with soil microbial resiliencies (biodiversity and functional diversity) after an anaerobic temporary event, most of them dealing with other media (sediments ...), geochemical conditions (microaerophilic) and without supplying wastes (van Bodegom et al., 2001).

The aims of this work were thus to (i) check the possibility of geochemical and microbial resilience of the soil over short period (2 d) after a return to aerobic condition, (ii) observe the fates of Fe and Mn over the same period, estimate the half life for their net immobilisation and model the involved processes, and (iii) describe the fates of mineral and organic small compounds that complex these metals and their effect on FeII and MnII mobilisation.

V.2 Materials and methods

V.2.1 Soil and organic liquid waste

Experiments were performed on a Ferralsol (FAO classification) sampled in Reunion Island at "La Mare" experimental station, 20°40' S and 55°32' E. Since 20 years, this experimental field has been used for sugarcane crop. The annual average precipitation is about 1500 mm with a potential evapotranspiration of 2300 mm. Sampling was performed on the 0-20 cm layer of the soil on the 3rd December 2002 after the harvest. The soil was then air-dried (9.67% residual moisture), stored in hermetic bags, and flown to Montpellier under cold conditions. It was mechanically crushed, sieved at 2 mm, and stored again in plastic bags at 4°C until the beginning of the experiments. Its properties were as follows: 679 g kg⁻¹ clay; 278 g kg⁻¹ silt; 44 g kg⁻¹ sand; 19.9 g kg⁻¹ organic C; 1.59 g kg⁻¹ organic N; 1.74 mg kg⁻¹ N-NH₄⁺; 6.16 mg kg⁻¹ N-NO₃⁻; 144.15 g kg⁻¹ total Fe, 3.20 g kg⁻¹ total Mn, 0.108 g kg⁻¹ total Cr, 0.963 g kg⁻¹ total Ni. Mobilised metals by DTPA extraction were: 24.78 mg kg⁻¹ Fe, 107.9 mg kg⁻¹ Mn, 0.40 mg kg⁻¹ Ni and Cr at concentration lower than 0.10 mg kg⁻¹.

The rum vinasse from molasses of sugarcane was collected at the low exit of a distillation column on the 10th December 2002. It was frozen at -20°C. Frozen vinasse was irradiated under γ -gamma source at 15 kGy, and stored at -20°C until experiments began. Dissolved Organic Carbon of the 1:10 diluted vinasse was 1.83 10⁻¹ mol C l⁻¹ and N concentration of alpha amino acid after HCl (6N) hydrolysis was 1.80 10⁻³ mol N l⁻¹. 10-fold diluted Vinasse contained 4.92 10⁻² mol C l⁻¹ of total carbohydrates, 4.62 10⁻³ mol C l⁻¹ or 1.22 10⁻³ mol N l⁻¹ total proteins, 1.05 10⁻² mol C l⁻¹ total phenols and other soluble organic matter including polyols, small organic acids, phenols and alcohols. The composition in main small carbohydrates, polyols, amino acids, organic acids and alcohols was measured by Ionic

Chromatography (IC) (section III.2.1, Table 1). Lipids were not found in the vinasse. The pH of 10-fold diluted vinasse was 4.98.

V.2.2 Experimental procedures

In order to characterize the fates of reduced Fe and Mn in liquid and solid phases, the fates of several mineral and organic ligands, and biodiversity changes during the aerobic period following an anaerobic incubation, the soil was initially supplied with the 10-fold diluted vinasse, then incubated during either 2, 7 or 21 d under anaerobic conditions, and finally incubated under aerobic conditions for 2 further days. Performed analyses deal with:

- i/ FeII and MnII concentrations in solution as well as FeII to FeIII ratio in solids in order to assess the kinetic of oxidation and precipitation of these metals, and the kinetics of dissolution of minerals containing FeII that appeared during the anaerobic period;
- ii/ concentrations of organic compounds (acetate, ...) and mineral ones (NH_4^+ , NO_2^- , NO_3^- , SO_4^{2-}) to assess the effect of their variation on metal mobilisation and speciation;
- iii/ microbial biodiversity that was indirectly assessed through PCR-SSCP profiles (Dassonville et al., 2004) to assess the possibility of microbial resilience on a short period.

V.2.2.1 Treatments and Measurements

In sealed 1 L flasks, 400 g of air dried soil were humidified 7 d before the beginning of the incubations with the addition of 70 ml of milliQ water. At the beginning of the experiments, the humidified soil was supplied with 600 ml of 10-fold diluted vinasse per flask. The slurries were then placed in anaerobic conditions, by alternating 3 successive cycles of 10 min vacuum and 10 min N_2 replacement. The last N_2 filling of the flask was considered as the initial time of the incubation (t_0). After a final equilibrium at atmospheric pressure, 1 ml of N_2 was replaced with Kr in order to calculate variations in total gas pressures. The flasks were then incubated at 25°C in darkness and continually shaken. Triplicate flasks for each of the following 3 treatments were defined according to the duration of the initial anaerobic incubation:

- V₂: 2 d of anaerobic incubation + 2 d of aerobic incubation;
- V₇: 7 d of anaerobic incubation + 2 d of aerobic incubation;
- V₂₁: 21 d of anaerobic incubation + 2 d of aerobic incubation.

At the end of the anaerobic period, aerobiosis was obtained by flushing the slurries with a 800 cm³.min⁻¹.flask⁻¹ flux of gas bubbles, made of 0.04 % CO₂, 20 % O₂, 79.96 % N₂. In order to avoid soil sedimentation in the flasks below the bubbling level, magnetic bar turning forced the turbulence in the bottom of the flask.

Along the anaerobic period, the atmosphere of the flask was characterized on triplicate flasks in order to check the variability of microbial activities between replicates and detect possible O₂ contamination. Measurements on the solution and the solid phase were done at the end of the anaerobic period and after 0.5, 3, 7, 24 and 48 h of aerobiosis. Approximately 120 ml of the slurry were first sampled from each of the 1 l flask replicates, ensuring the solution to solid mass ratio is approximately the same in the 1 l flask and the sample aliquot that was stored in 150 ml flasks. Measurements on the solution were performed on the 3 150 ml replicate flasks sampled at each date. They dealt with:

- the concentrations of total carbohydrates, total proteins; and identified organic compounds (carbohydrates, polyols, small organic acids and alcohols);
- the concentrations in mineral anions and cations (SO₄²⁻, Cl⁻, NO₃⁻, NO₂⁻, NH₄⁺, Ca²⁺, Mg²⁺, K⁺, Na⁺, Al³⁺, Zn²⁺, Cu²⁺);
- total metal (Fe, Mn) concentrations, and FeII concentration;
- pH and E_H.

Microbial analyses and measurements of the Fe oxidation level in the solid phase were performed at the end of anaerobic and aerobic periods on only 1 flask sampled per date and treatment. They dealt with:

- the FeII to FeIII ratio of the solid phase (Trolard et al., 1997)
- the evolution of microbial diversity using PCR-SSCP methodology (Dassonville et al. 2004).

Nous ne présentons actuellement les mesures que pour le sol initial, le sol incubé pendant 21 jours en anaérobiose et le sol incubé pendant 21 jours d'anaérobiose suivis de 2 jours d'aérobiose. Les mesures concernant les autres traitements doivent être recommencées avant soumission de l'article, suite à un mauvais conditionnement des échantillons pour le Mössbauer;

Gas measurements (O_2 , CO_2 , N_2 , N_2O , H_2 , CH_4) were performed on a TCD gas chromatograph (CP 3800, Varian, USA) fitted with Porapak Q (80-100 μm mesh, 1.8 m) and sieve molecular (1-5 \AA , 1.8 m) columns coupled to an integration software, Star WS. Carrier gas was He. Relative uncertainties were about 1% (3% for H_2). Before gas sampling, the flasks were vigorously shaken to ensure equilibrium between gas and liquid phases. 0.20 ml of gas was then sampled with a syringe and analysed by TCD chromatography.

To prevent oxidation of the media and pH variation due to CO_2 volatilisation, the pH and the redox potential E_H were measured as soon as the flasks of set 2 were opened under N_2 atmosphere in a glove bag using pH (Xerolyt, HA405-DXK-S8/120, Mettler Toledo, N.Y., U.S.A.) and E_H electrodes made of platine reference electrode and Ag/AgCl electrolyte (Pt4805-DXK-S8/120, Mettler Toledo, N.Y., U.S.A.). The measurements were performed on the supernatant.

For all flasks, the soil solution handling was performed under strict N_2 atmosphere, as well as the centrifugation that was operated during 15 min at 1800 g at 4°C (Heraeus, Biofuse stratus, Germany), the filtration at 0.22 μm (Millex GP, Millipore, France), and the distribution of the solution in sterile tubes. The measurement of FeII concentration was immediately done. Tubes intended for the measurement of organic compounds were supplied with NaN_3 to obtain 30 mg l⁻¹ concentration. Tubes intended to measure total metal concentrations were supplied with 25 μl concentrated HCl in order to avoid oxy-hydroxide precipitation. No chemical was added to the tubes intended for $N-NO_3^-$, $N-NO_2^-$ and $N-NH_4^+$ analyses. All the tubes were then stored at 4°C until measurements.

Colorimetric methods (Jasco V-530, UV-VIS Spectrophotometer, Tokyo, Japan) were used for the determination of total carbohydrates at 627 nm (Dubois et al., 1956) and total protein at 595 nm (Bradford, 1976).

The concentrations in small organic compounds were determined by IC (Dionex DX 600, U.S.A.) fitted with (i) both CARBOPAQ-PA1 and CARBOPAQ-MA1 columns with a detector of pulsed amperometry for sugars and polyols, (ii) a CARBOPAQ-MA1 column and a detector of pulsed amperometry for alcohols, (iii) an IONPAQ-AS11 column and a conductimetric detector for organic acids and mineral anions, such as NO_3^- , SO_4^{2-} , Cl^- and

PO₄³⁻. However, we have retained measurements of NO₃⁻, NO₂⁻ and NH₄⁺ performed by spectroscopy (Alliance, EvolutionII, France).

Total concentrations in Fe and Mn in solution were determined by ICP-AES (Varian, VISTA-Pro, CCDsimultaneous, France), whereas DPKBH was used as a reagent to determine FeII concentration by spectrophotometry at 660 nm (Bourri  et al., 1999).

⁵⁷Fe M ssbauer measurements were performed at 75 K with an EG and G constant acceleration spectrometer in transmission mode. The   source of nominal activity 10 mCi was ⁵⁷Co in a Rh matrix. The velocity scale (  10 mm/s) was calibrated with reference to the magnetic sextet spectrum of a metallic iron foil absorber and all isomer shifts are given with respect to the centre of this spectrum. After incubation and additional treatments the absorbers were prepared inside a glove bag under N₂ atmosphere, from approximately 500 mg of samples and introduced in specific lead sample holder (10 mm diameter, 2 mm thick), tight and transparent to the   rays. This cell was composed of two very thin 14 mm diameter Mylar windows separated by 2 mm of lead. Experimental data were analysed using the ISO software (K nding, 1969) by fitting the recorded spectra to Lorentzian profiles by the least squares method. Goodness of fit was controlled by the classical “chi-squared” test.

Single-strand conformation polymorphism (SSCP) analysis relies on the fact that a single modification can change the conformation of a single strand DNA molecule, leading to a different electrophoretic mobility in a non-denaturing gel (Orita et al., 1989). In this way, DNA fragments of the same size but different base compositions can be separated (Clapp, 1999). When applied to 16S rDNA fragments from a microbial community, SSCP analysis gives a pattern of peaks, while analysis of pooled 16S rDNA from a single fragment gives one peak. DNA extractions were first performed on three replicate flasks at each sampling date, accordingly to the method described in Dassonville et al. (2004) on 10 ml sample (mixture of soil and solution) from each flask. Then, the variable V3 region (Escherichia coli positions 331–533) was then amplified using the W91–94 pairs of primers (Dassonville et al., 2004). We performed finally a PCR-based SSCP analysis using an automated DNA sequencer (ABI 310 Genetic Analyzer, Applied Biosystems) equipped with a capillary tube (47 cm   50 mm) filled with a polymer composed of GeneScan polymer (Applied Biosystems) 5.6%, glycerol 10% and TBE 1  , using the method of Dassonville et al. (2004). *Les r sultats de ces analyses mol culaires ayant  t  acquis r cemment, quelques uns seront pr sent s   l’ tat brut sans analyse approfondie. Ils seront repris avant la soumission de l’article.*

V.2.3 Data treatments

V.2.3.1 Metal speciation in solution

Speciation calculations aimed to assess (i) the contributions of mineral and organic compounds – that are microbially produced and/or consumed – to the complexation and mobilisation of metals, and (ii) the effect of the return to aerobic conditions on these compounds and the resulting FeII and MnII speciations. Speciation calculations were performed at the end of the anaerobic phase in V₂, V₇ and V₂₁ treatments, and completed by calculations of saturation indices for potentially precipitating minerals to evaluate possibility of precipitations. Chemical speciation in solution was indirectly assessed using GEOCHEM-PC (Parker et al., 1995). This geochemical model enables to account for equilibria in solution of simple ‘metal-ligand’ complexes, and the precipitation/dissolution of solids. The database of equilibrium constants combined the database of Parker et al. (1995) and additional constants issued from the NIST standard reference database 46 version 7.0 for the following ligands: propionate, lactate, butyrate, pyruvate, cisaconitate, transaconitate. At the end of anaerobic phase of V₂, V₇ and V₂₁ treatments, calculation of saturation indices for potentially precipitating mineral phases were performed using solution data and compared with the corresponding pKs values found in literature (Stumm et Morgan, 1996; Génin, 1998). Saturation indices were performed for several FeII, MnII and mixed FeII-FeIII minerals: magnetite, green rust carbonate, green rust chloride, green rust sulfate, siderite, rhodocrosite, vivianite, maghemite, pyrrhotite, alabandite.

V.2.3.2 Half life for FeII net immobilisation and a dimensionalisation of FeII concentrations

We used an empirical exponential model to characterize the time scale of FeII oxidation and immobilisation:

$$\hat{FeII}_{aq}(t) = \hat{FeII}_{aq}(t=0) \times e^{-k \times t} \quad (1)$$

where $\hat{FeII}_{aq}(t)$ is the simulated concentration in FeII in solution (mol.l^{-1}) at time t (s), and k and empirical constant (s^{-1}). Simulated $\hat{FeII}_{aq}(t)$ data were fitted to experimental $FeII_{aq}(t)$ ones by minimizing the following expression, using the Marquardt–Levenberg algorithm:

$$\chi^2 = \sum_t (\hat{FeII}_{aq}(t) - FeII_{aq}(t))^2 \quad (2)$$

The resulting estimate of k enable to easily estimate the half life $t_{1/2}$ (s) of $\hat{FeII}_{aq}(t)$:

$$t_{1/2} = \frac{\ln 2}{k} \quad (3)$$

The $t_{1/2}$ constant was estimated independently for each of the 3 treatments V₂, V₇ and V₂₁ ($\hat{t}_{1/2}(2)$, $\hat{t}_{1/2}(7)$ and $\hat{t}_{1/2}(21)$ estimates, respectively).

Assuming that the oxidation and immobilisation of FeII exists only in solution and is then proportional to the experimental FeII concentration $FeII_{aq}(t)$, the $FeII_{aq}(t)$ to $FeII_{aq}(t=0)$ ratio should then be independent of the treatment as long as there is no immobilised FeII. Comparing the functions for the 3 treatments V₂, V₇ and V₂₁ enabled to check this hypothesis.

V.2.3.3 Mechanistic model of Fe fate

The model accounts for the dissolution of a pool of easily soluble FeII, and the oxidation of FeII in solution. The abiotic oxidation of FeII was assumed to depend only on $FeII_{aq}(t)$:

$$\phi_o(t) = -k_o \times FeII_{aq}(t) \quad (4)$$

where $\phi_o(t)$ is the flux of oxidised FeII in solution ($\text{mol.s}^{-1}.\text{l}^{-1}$), from which most precipitates as FeIII oxy-hydroxide, and k_o is a constant of oxidation (s^{-1}). We assumed that the dissolution of FeII minerals (i) is proportional to their surface that would vary proportionally to their mass with exponent 2/3 as spheres, and (ii) does not depend on $FeII_{aq}$, i.e. $FeII_{aq}$ is far from equilibrium with easily soluble FeII minerals, due to the changes with the return to aerated conditions (including a decrease in CO₂ partial pressure and an increase in pH):

$$\phi_d(t) = k_d \times (FeII_s(t))^{2/3} \quad (5)$$

where k_d is a constant of dissolution, $FeII_s$ (mol l^{-1}) is the amount of FeII in solids expressed per unit volume of solution (mol.l^{-1}). Solving equations (4) and (5) with a numerical method

enabled us to simulate $\hat{FeII}_{aq}(t)$ and $\hat{FeII}_s(t)$. Since Mössbauer data are available only for the initial soil, the soil after 21 d of anaerobic incubation and also after 2 additional days of aerobic incubation, and since additional simulations showed that it was possible to fit experimental $FeII_{aq}$ only if easily soluble FeII totally disappeared during the 2 days of aerobic bubbling (results not shown), we estimated the easily soluble FeII pool for the V₂₁ treatment using Mössbauer data, and assume a linear increase in this pool during the initial aerobic incubation starting from an unknown date d_{pi} (d), using the following equation that are valid only for the initial anaerobic incubation:

$$FeII_s(n) = FeII_s(21) \times \left(\frac{n - d_{pi}}{21 - d_{pi}} \right) \quad \text{when } n > d_{pi} \quad (6a)$$

$$FeII_s(n) = 0 \quad \text{when } n \leq d_{pi} \quad (6b)$$

Where n is the duration of the anaerobic incubation (d).

Simulations thus require to estimate 3 parameters: k_o , k_d , and d_{pi} . Practically, we first estimates simultaneously k_o and k_d , by fitting simulated $\hat{FeII}_{aq}(t)$ to experimental $FeII_{aq}(t)$, using the Marquardt–Levenberg algorithm, for treatment V₂₁ (FeII_s pool just after the return to aerobiosis is known). Then, we used the obtained estimates of k_o and k_d to estimate only d_{pi} by fitting simultaneously simulated $\hat{FeII}_{aq}(t)$ to experimental $FeII_{aq}(t)$ for treatments V₂ and V₇.

V.3 Results

V.3.1 Changes during the initial anaerobic period

Total carbohydrate concentrations decreased by 68.5, 88.1 and 91.2 % at the end of the anaerobic phase of V₂, V₇ and V₂₁ treatments (Fig 1), respectively. Only traces of polyols from the vinasse inputs were detected at the end of 3 anaerobic incubations. No protein were detected after 2, 7 and 21 d of anaerobiosis. Main organic acids in the vinasse were acetate, formate and lactate, whereas no butyrate and propionate were detected. Formate ($2.69 \cdot 10^{-4} \text{ mol l}^{-1}$) decreased by 79.9, 69.6 and 56.8 % at the end of V₂, V₇ and V₂₁ anaerobic periods, respectively. Acetate increased from $8.53 \cdot 10^{-4} \text{ mol l}^{-1}$ to $1.45 \cdot 10^{-2}$, $2.60 \cdot 10^{-2}$ and $2.91 \cdot 10^{-2}$

2 mol l^{-1} after 2, 7 and 21 d of anaerobiosis, respectively (Fig 1). Butyrate achieved a peak concentration ($3.58 \cdot 10^{-3} \text{ mol l}^{-1}$) after 2 d of incubation and was equal to $1.65 \cdot 10^{-3}$ and $2.50 \cdot 10^{-4} \text{ mol l}^{-1}$ after 7 and 21 d of anaerobiosis, respectively. Lactate ($6.89 \cdot 10^{-4} \text{ mol l}^{-1}$ at t_0) was not detected at any end of anaerobic incubation, whereas propionate was produced to $6.67 \cdot 10^{-4}$, $3.47 \cdot 10^{-3}$ and $3.86 \cdot 10^{-3} \text{ mol l}^{-1}$ at the end of V_2 , V_7 and V_{21} anaerobic period,

Figure 1: Main organic compound and CO_2 concentrations at the end of anaerobiosis for each

treatment.

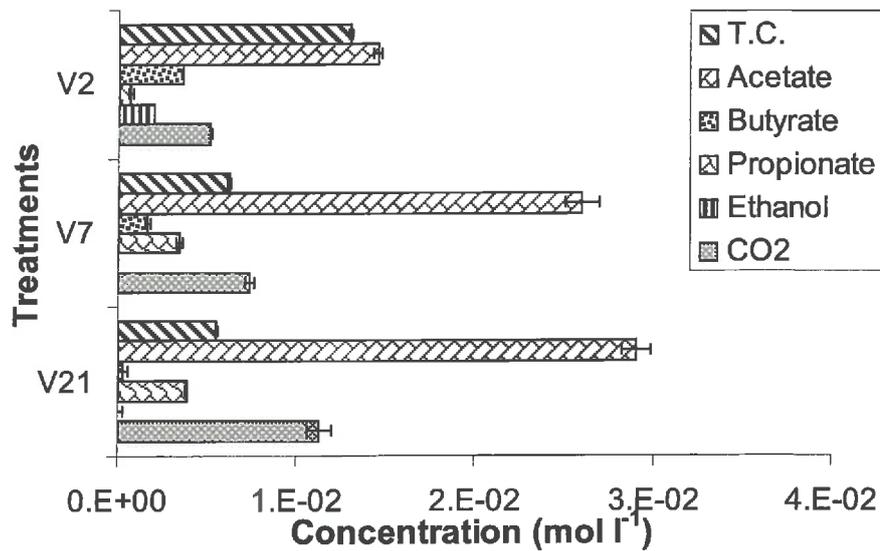
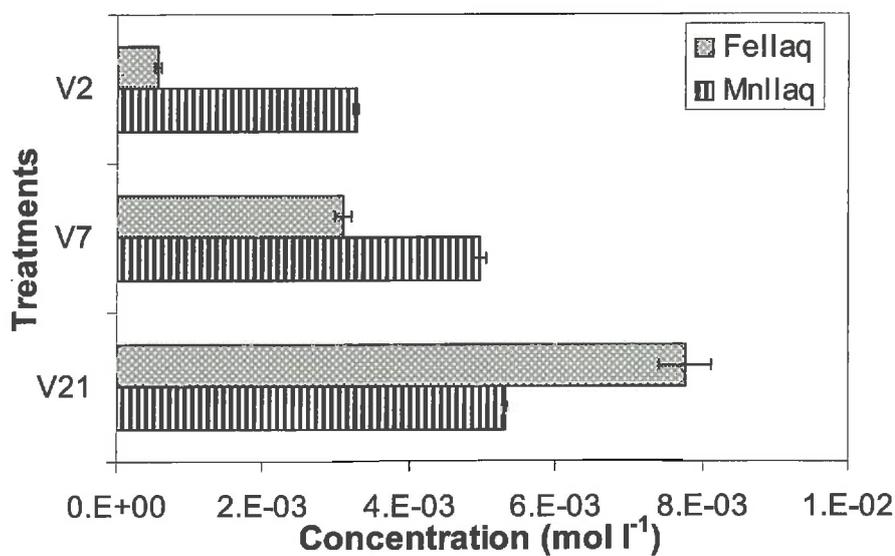


Figure 2: Mobilised Fe and Mn after 2, 7 and 21 d of anaerobiosis.



respectively (Fig 1). Other organic acids ($1.09 \cdot 10^{-3} \text{ mol C l}^{-1}$ initially) decreased by 57.7 % after 2 d of anaerobiosis and were all consumed at the end of longer anaerobic incubations. No ethanol was detected in the vinasse. It appeared after 2 and 7 d of anaerobic phase to respective concentration of $6.20 \cdot 10^{-3}$ and $5.05 \cdot 10^{-7} \text{ mol l}^{-1}$, respectively (Fig 1).

CO_2 partial pressure greatly increased during the first 7 d of incubation ($2.14 \cdot 10^{-1} \text{ atm}$); it thereafter stabilised until 21 d of incubation, to $2.76 \cdot 10^{-1} \text{ atm}$. No significant emission of CH_4 was observed at any date of anaerobiosis. An H_2 peak was detected between 7 h and 2 d of incubation, reaching a maximum value of $2.64 \cdot 10^{-2} \text{ atm}$ at 1 d. A peak of N_2O ($2.19 \cdot 10^{-4} \text{ atm}$) was observed after 5 h of incubation in the treatments and disappeared at 1 d.

Nitrate ($5.79 \cdot 10^{-4} \text{ mol l}^{-1}$ initially) was not detected after 2, 7 and 21 d of anaerobiosis. SO_4^{2-} concentration ($1.35 \cdot 10^{-3} \text{ mol l}^{-1}$) decreased of 23.7, 96.8 and 98.2 % at end of V_2 , V_7 and V_{21} anaerobic phase, respectively. NH_4^+ concentration in solution ($3.52 \cdot 10^{-5} \text{ mol l}^{-1}$ initially) decreased of 68.2 % during the 2 first days of anaerobiosis, and increased then to reach 5.2- and 15.7-time the initial concentration after 7 and 21 d of anaerobiosis, respectively.

pH increased from 5.38 to 6.24, 5.98 and 5.90 in treatments V_2 , V_7 and V_{21} , respectively. E_H globally increased from 180.0 to 119.5 mV in V_2 treatment, whereas it decreased to 13.2 and 12.1 mV at the end of V_7 and V_{21} treatments, respectively.

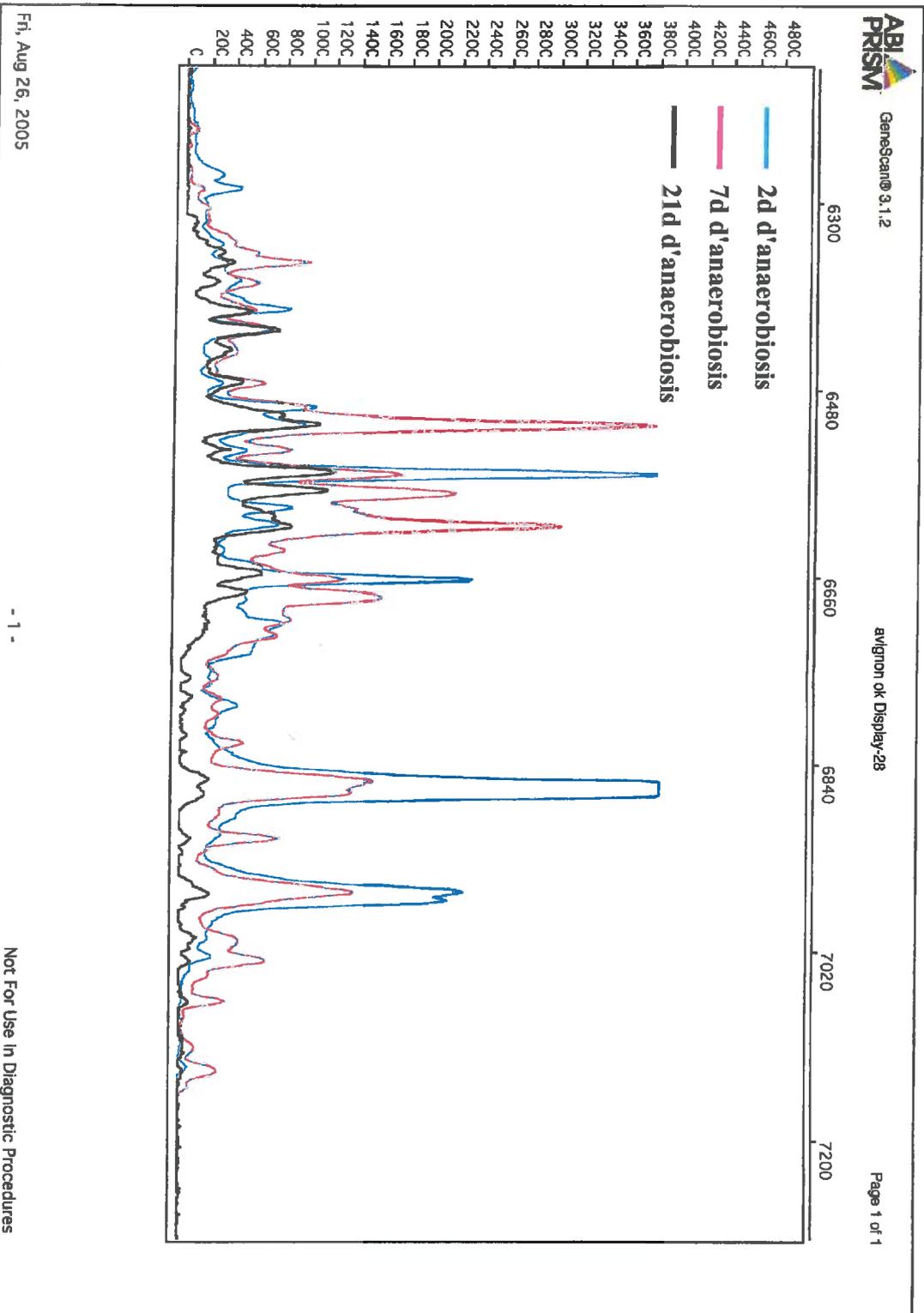
Initially, no FeII and MnII were detected in solution and FeII in solids represented 1.50 % of total FeII in solids (corresponding to $4.15 \cdot 10^{-3} \text{ mol FeII g}^{-1}$ dry soil). Mobilised Fe and Mn were equal to $5.54 \cdot 10^{-4}$ and $3.27 \cdot 10^{-3}$, $3.39 \cdot 10^{-3}$ and $4.95 \cdot 10^{-3}$, $7.77 \cdot 10^{-3}$ and $5.31 \cdot 10^{-3} \text{ mol l}^{-1}$, after 2, 7 and 21 d of anaerobic incubations, respectively (Fig 2). Table 1 sums up the speciation of mobilised FeII and MnII in solution at the end of the anaerobic period of each treatment. For all dates, FeII and MnII were mainly as free cations in the soil solution. Determination of log IAP (results not shown) indicated value higher than log K_S for the minerals FeS, MnS, after 2, 7 and 21 d of anaerobiosis and FeCO_3 and MnCO_3 for the last 2 dates. No FeII and MnII hydroxides were supposed to form according to IAP calculations at any date. In fact, Mössbauer spectroscopy detected $6.78 \cdot 10^{-2} \text{ mol FeII g}^{-1}$ dry soil after 21 d of incubation, from which about $1.66 \cdot 10^{-2} \text{ mol FeII g}^{-1}$ dry soil were FeII similar to FeII in green rust. Consequently, the ratio of FeII in solution versus FeII in solid was equal to 2.42 % after 21 days of anaerobic incubation.

Nous présentons quelques spectres de PCR-SSCP dans la Figure 3. Ces données feront l'objet d'analyses ultérieures à la thèse avant soumission de l'article.

Table 2 : Speciation of mobilised Fe and Mn at the end of the anaerobic phase for the three treatments.

| Treatment | Ligands | Speciation FeII _(aq) (%) | Speciation Mn _(aq) (%) |
|-----------------|--|--|--------------------------------------|
| V ₂ | Free cations | 83.6 | 78.7 |
| | Organic acids | 14.6 | 12.2 |
| | Mineral anions without CO ₃ ²⁻ | 1.8 | 4.5 |
| | CO ₃ ²⁻ | 0.0 | 4.6 |
| V ₇ | Free cations | 81.7 | 73.5 |
| | Organic acids | 18.3 | 17.9 |
| | Mineral anions without CO ₃ ²⁻ | 0.1 | 2.0 |
| | CO ₃ ²⁻ | 0.0 | 6.6 |
| V ₂₁ | Free cations | 81.1 | 64.2 |
| | Organic acids | 18.9 | 16.3 |
| | Mineral anions without CO ₃ ²⁻ | 0.01 | 1.9 |
| | CO ₃ ²⁻ | 0.0 | 17.5 |

Figure 3: PCR-SSCP profiles after 2, 7 and 21 d of anaerobiosis.



V.3.2 Experimental changes after a return to aerobic conditions

V.3.2.1 Fe and Mn fates

MnII concentrations decreased during the first 0.5 h of aerobiosis by 15, 8 and 24 % for treatments V₂, V₇ and V₂₁, respectively. They stabilised thereafter or slightly increased (Fig 4b). FeII concentrations in solution decreased along the aerobic phase for the 3 treatments to reach nearly 0 values after less than 24 h incubation, whatever the treatment (Fig 4a). FeII in solids decreased from $6.78 \cdot 10^{-2}$ to $5.12 \cdot 10^{-2}$ mol g⁻¹ dry soil (24.5 % of decrease) along the 2 d of aerobiosis in V₂₁ treatment. At the end of aerobiosis FeII_(aq)/FeII_(s) corresponded to 0.02 %.

The dimensionalisation of *FeII*_{aq} by dividing measured values in aerobiosis with the value at the beginning of aerobiosis in the same treatments led to experimental data that don't merge between treatments (Fig. 4a). More precisely, the decreasing of the *FeII*_{aq} (t) to *FeII*_{aq} ((t=0)) ratio is all the more slow as the initial anaerobic incubation was long.

Oxidation constants of *FeII*_{aq} were defined on the basis of Eq. 1 using an exponential empirical model of *FeII*_{aq} variation with time t (Table 3).

V.3.2.2 Other geochemical changes in the slurry solutions

Total carbohydrates along V₂ aerobic incubation decreased by 46.25 % (i.e. corresponding to a decrease of $6.03 \cdot 10^{-3}$ mol C l⁻¹); simultaneously, the decrease in small sugars was of 76.7 % (decrease of $5.98 \cdot 10^{-4}$ mol C l⁻¹) during the same period. During the aerobic phase of V₂ treatment, proportion of small sugars (C₅, C₆, C₁₂) among total carbohydrates decreased from 6.0 to 2.6 %. In V₇ treatment, total carbohydrates and small sugars slightly decreased by 6.6 % and 7.2 % (i.e. corresponding to a decrease of $4.13 \cdot 10^{-4}$ and $5.84 \cdot 10^{-6}$ mol C l⁻¹), respectively. During V₇ aerobic period, the proportion of small sugars fluctuated between 1.2-1.8 % of total carbohydrates. In V₂₁ treatment, total carbohydrates increased by 6.6 % (i.e. corresponding to an increase of $3.64 \cdot 10^{-4}$ mol C.l⁻¹), concomitantly to an increase of small sugars of 47.5 % (increase of $7.57 \cdot 10^{-6}$ mol C l⁻¹). In this treatment, the contribution of summed small sugars to total carbohydrates ranged into

Figure 4: Reduced Fe (a) and Mn (b) evolution after a switch to aerobiosis.

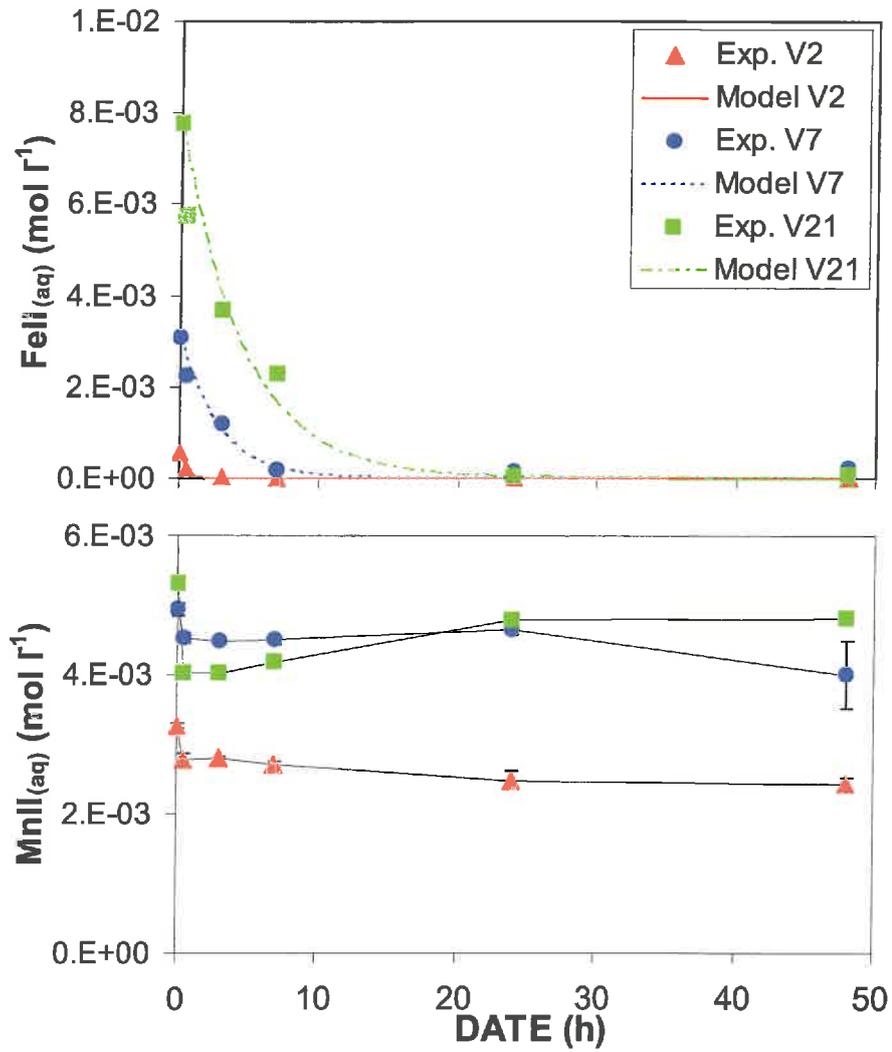


Table 3: Oxidation constants and dissolution defined by numerical model.

| Treatment | Model 1 FeII(aq) oxidation constant s^{-1} | Model 1 $T_{1,2}$ (h) |
|-----------------|---|-----------------------------|
| V ₂ | $5.56 \cdot 10^{-4}$ | 0.35 (20.8 min) |
| V ₇ | $1.02 \cdot 10^{-4}$ | 1.88 |
| V ₂₁ | $6.27 \cdot 10^{-5}$ | 3.07 |

0.3-0.7 % range along the 2 d of aerobiosis. Organic acid did not show important changes in concentration along the aerobic phase, whatever the treatment. Acetate concentrations were stable along the 2 d of aerobiosis for the 3 treatments (Fig 5). Butyrate decreased in all treatments during aerobiosis incubation (Fig 5). Propionate concentration was globally stable for the three treatments (Fig 5).

SO_4^{2-} concentrations increased during the first 0.5 h of aerobiosis and then stabilise in treatment V_2 and V_7 (Fig 6). In contrast, it was stable with a slight increase at the end of the V_{21} treatment. NH_4^+ concentration globally decreased even if a peak was observed after 24 h of aerobiosis in V_2 ($1.72 \cdot 10^{-5} \text{ mol l}^{-1}$) and V_{21} treatments ($7.17 \cdot 10^{-4} \text{ mol l}^{-1}$) (Fig. 6).

pH increased during the first 0.5 h of aerobiosis for the 3 treatments, and then globally slightly increase for V_2 and decreased for V_7 and V_{21} (Fig 7). E_H evolutions are presented in Figure 7.

V.3.2.3 Biodiversity evolution over the anaerobic/aerobic incubation periods

Nous présentons quelques spectres de PCR-SSCP dans la Figure 8. Ces spectres présentant l'évolution de la population microbienne dans un sol incubé sous anaérobiose pendant 7 jours et un sol incubé en anaérobiose pendant 7 jours suivi de 2 jours d'aérobiose. Des spectres ont ainsi été obtenus après 2, 7 et 21 jours d'anaérobiose et en fin des 2 jours d'aérobiose pour ces 3 traitements. Ces données feront l'objet d'analyses ultérieures à la thèse avant soumission de l'article.

V.3.3 Simulated fate of FeII after a return to aerobic conditions

Fitting simulated \hat{FeII}_{aq} to experimental ones $FeII_{aq}$ during the 2 days of aerobic incubations in treatment V_{21} leads to a good agreement between the model and the experiment of the (Fig. 9c). The estimations of k_o and k_d were equal to $7.24 \cdot 10^{-6} \text{ s}^{-1}$ and $4.64 \cdot 10^{-5} \text{ s}^{-1}$, respectively. Using these constants to estimate d_{pi} parameter by fitting simulated data to experimental ones in treatments V_2 and V_7 led to a good agreement between simulations and experiments (Fig. 9a,b). d_{pi} estimate was equal to 2.92 d, i.e. the model did not consider FeII in solids for the V_2 treatment.

Figure 5: Main organic acid evolutions after a return to aerobic conditions.

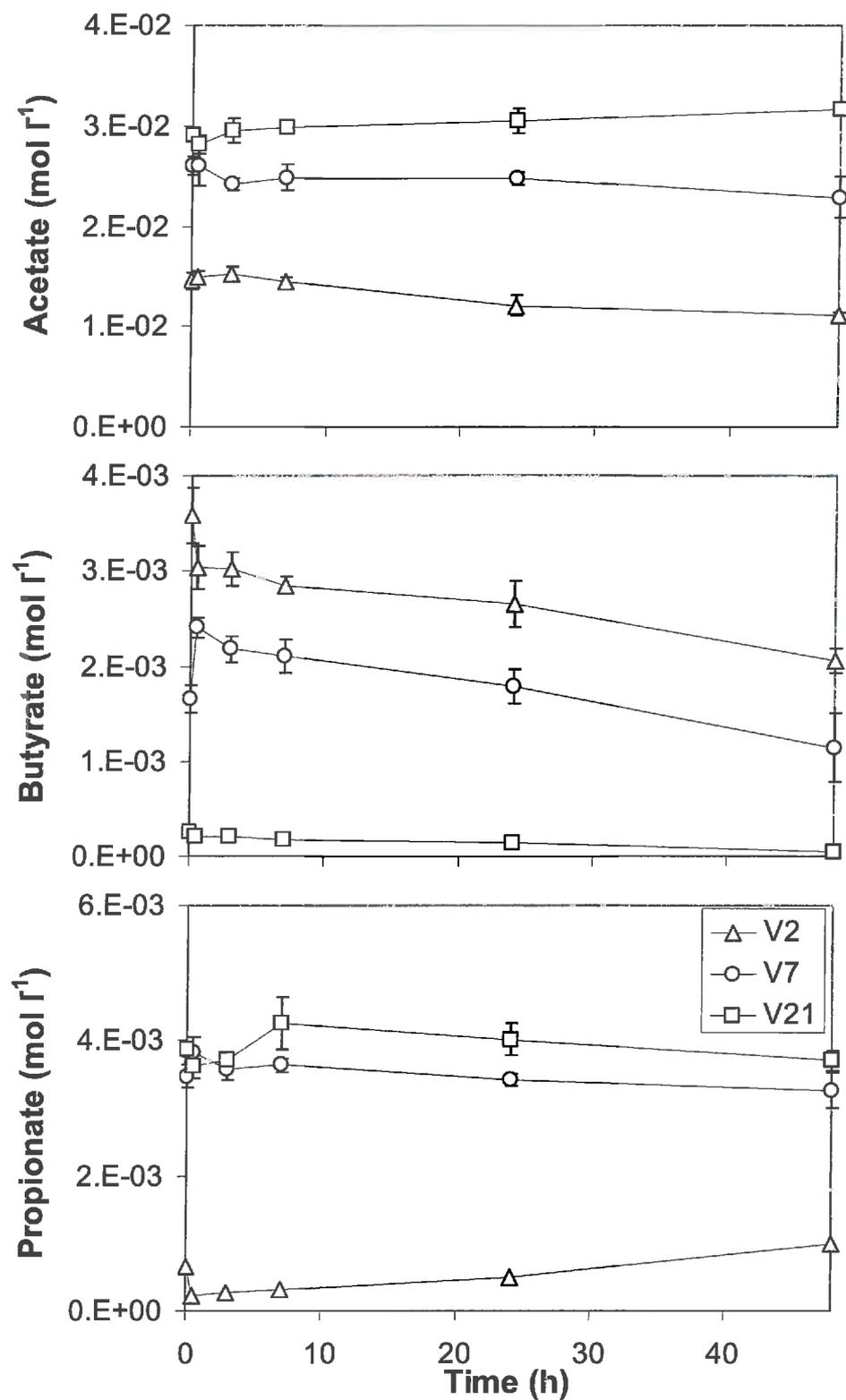


Figure 6: SO_4^{2-} and NH_4^+ evolutions along 2 d of aerobiosis.

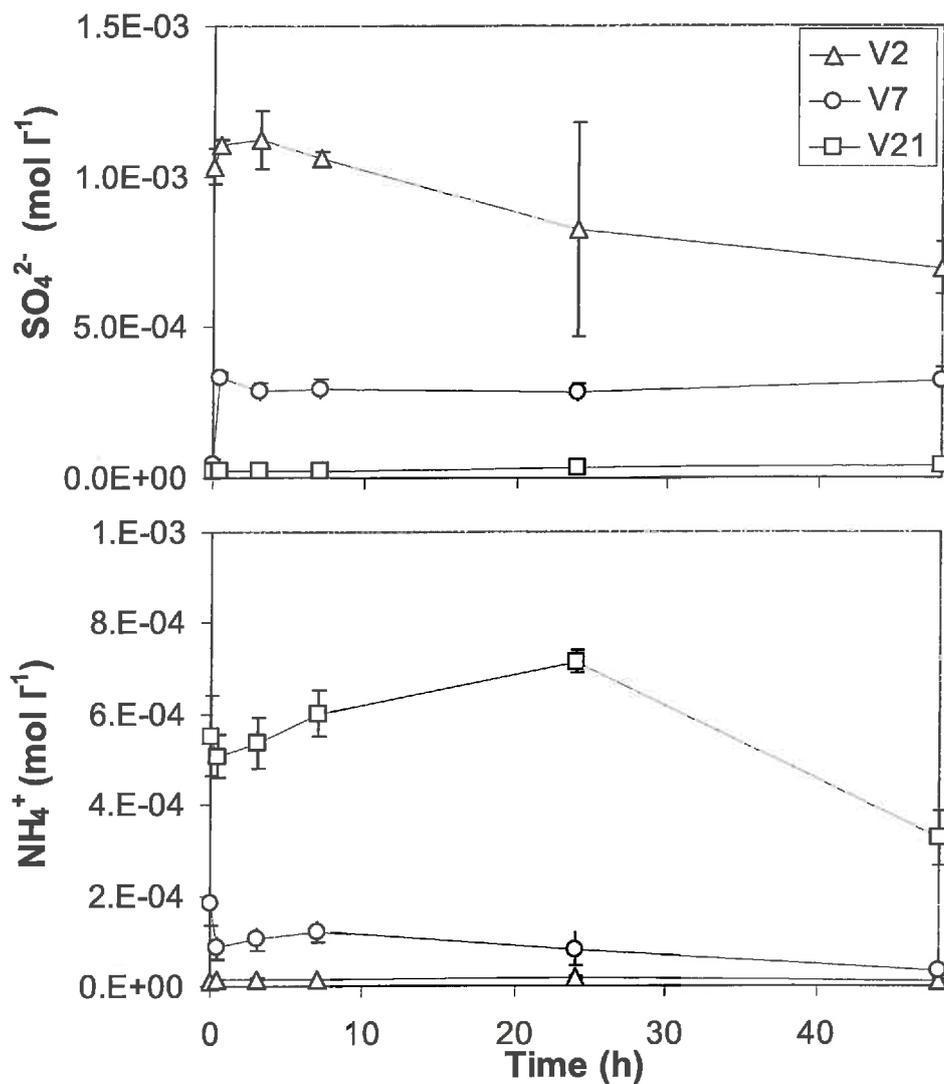


Figure 7: pH and E_H evolutions after a return to aerobic conditions.

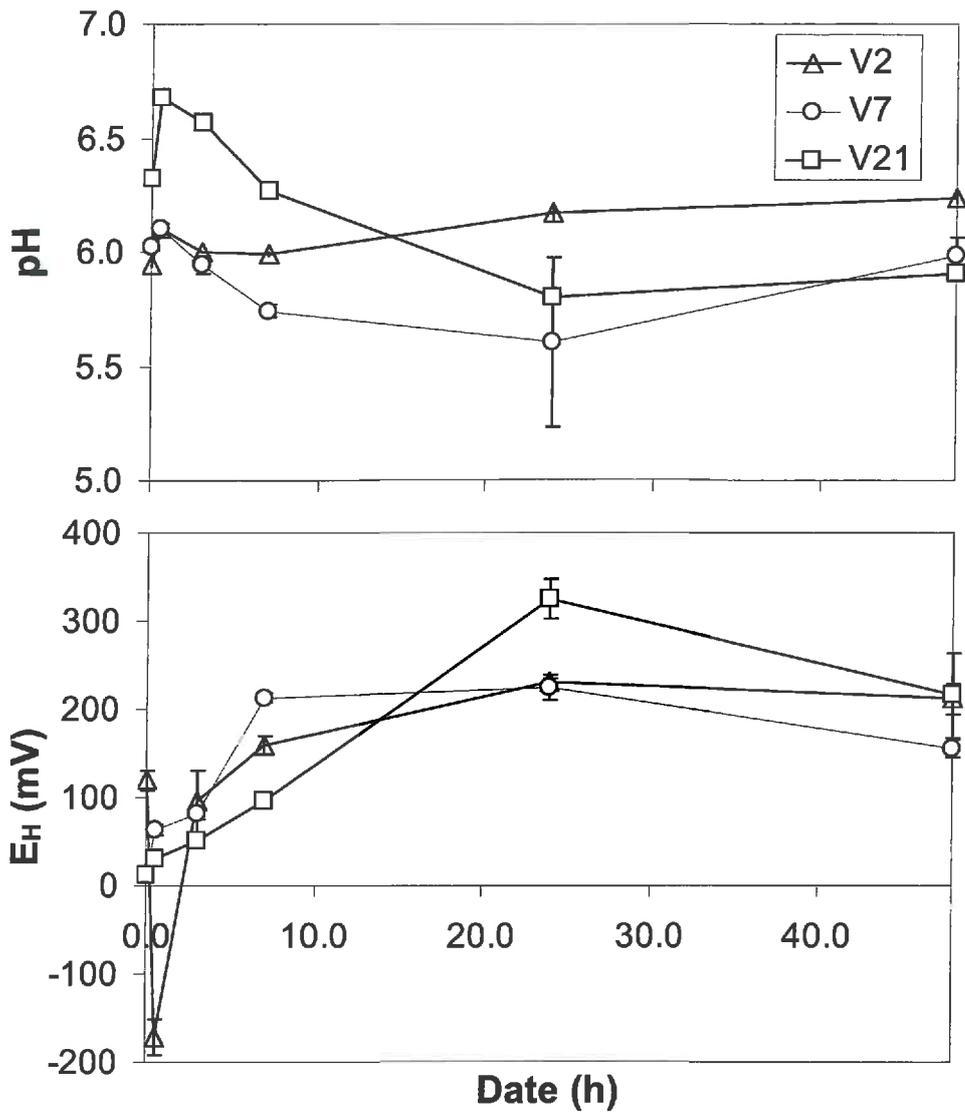


Figure 8: PCR-SSCP profiles of soil amended with rum vinasse after 7 d of anaerobiosis and 7 d of anaerobiosis + 2 d of aerobiosis.

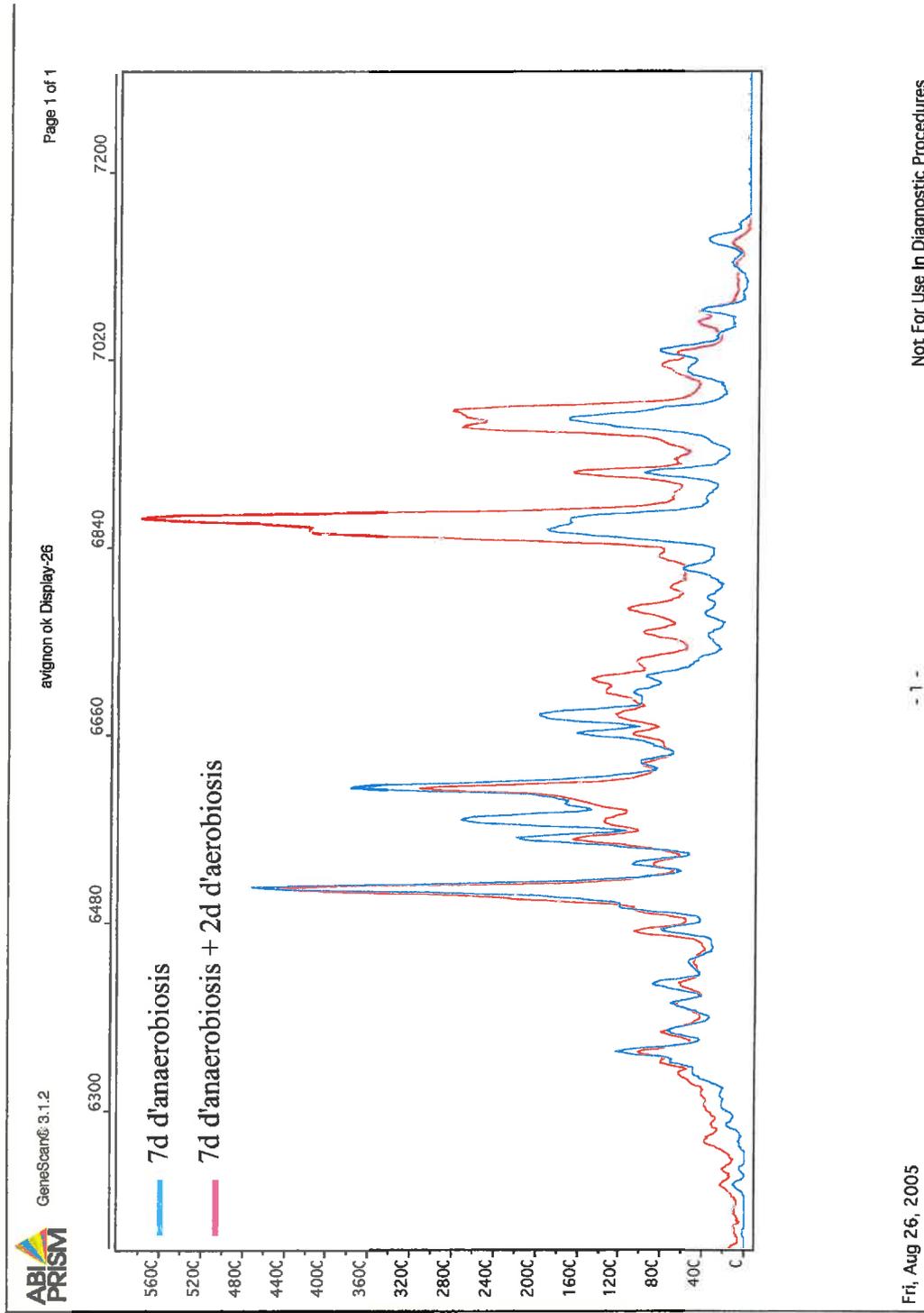


Figure 9: Simulated fate of FeII after return to aerobiosis.

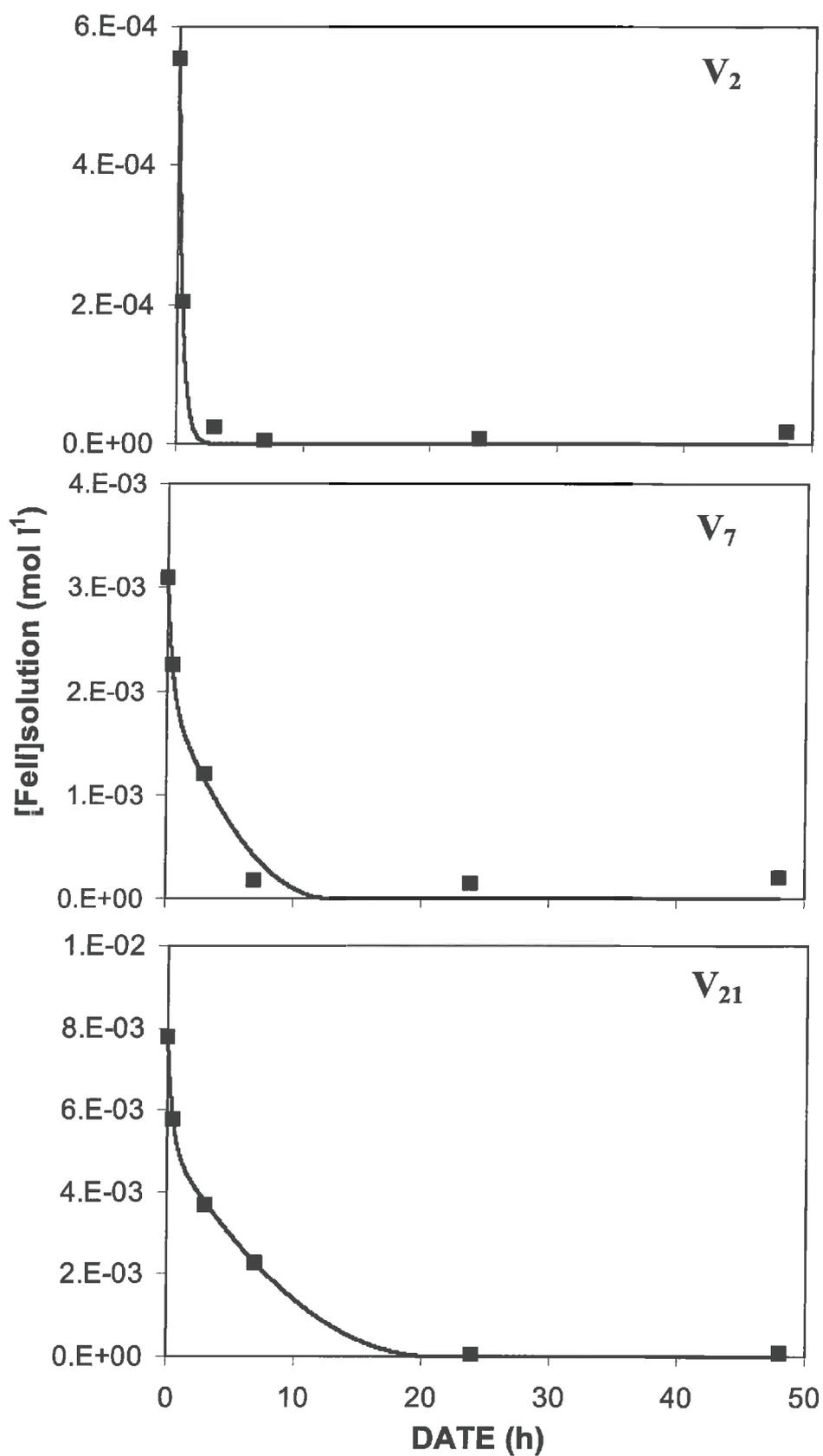
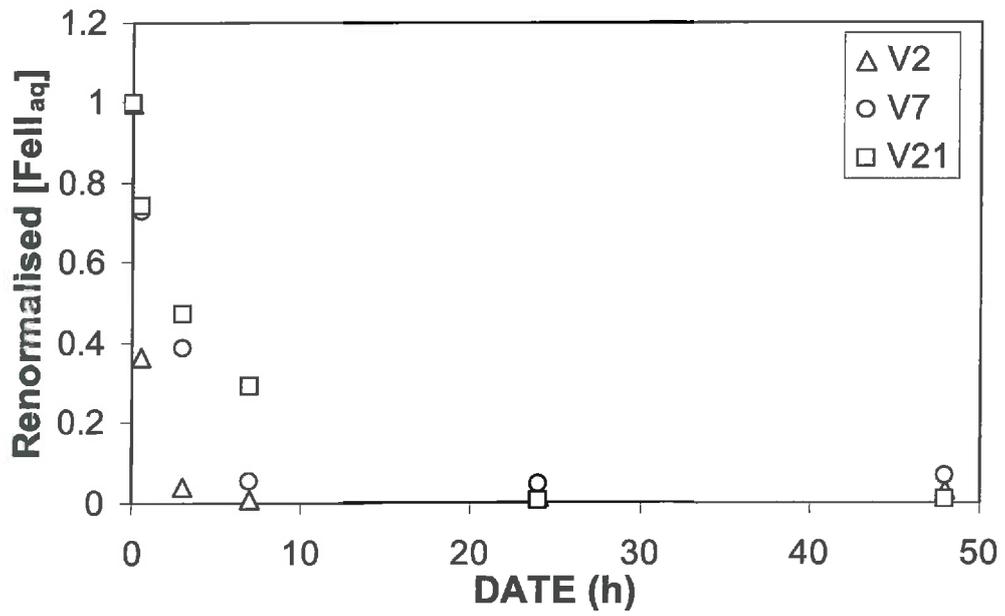


Figure 10: Renormalisation of FeII after return to aerobiosis.



V.4 Discussion and Conclusions

V.4.1 Fates of FeII and MnII after the return to aerobic conditions

Reduced Mn and Fe evolutions under aerobic conditions were not correlated, as we could expect from literature (Tebo and He, 1999).

More precisely, Fe oxidation and immobilisation in solution were total after a few hours, as it can be seen in treatment V₂, in which there was a priori no solid FeII (Fig. 9a). The rate of this process confirmed its abiotic nature, as it is generally recognised in the literature (Stumm and Morgan, 1996). However, FeII immobilisation more generally depends on its simultaneous dissolution and oxidation, as discussed in others studies (Morgan, 2005; Duckworth and Martin, 2004, Neal et al., 2001); the existence of a pool of FeII in solids, that is easily dissolved, increases then the time required for mobilised FeII to disappear. This can be seen from Fig. 10 and from half life of FeII immobilisation that increased in the order V₂ < V₇ < V₂₁. (0.35 h, 1.89 h and 3.07 h, respectively) (Table 3). This can also be seen from Fig. 9a-c. Using similar approaches as in Eq. (1) for oxidation of FeII in solution, Katsoyannis et Zouboulis (2004) estimated constant k for oxidation of about 1.17 10⁻² s⁻¹ for groundwater in presence of Mn oxides and *Gallionella* and *Leptothrix* bacteria (i.e. that combined microbial and abiotic processes), whereas works on exclusively abiotic oxidation of FeII for groundwater or sea water led to constants k in the range 5.79 10⁻⁶ to 1.85 10⁻³ s⁻¹. (Murase et Kimura, 1997; Ratering et Conrad, 1998; van Cappellen et Wang, 1996). Our estimate (7.24 10⁻⁶ s⁻¹) is among the lowest estimates. The possible reasons to explain our low oxidation constant include the acid pH values of our slurries (5.6 – 6.7) and a negligible microbial contribution.

Saturation calculations in the soil solution indicated possible precipitation of FeCO₃ and FeS at the end of the anaerobic phase for the 3 treatments V₂, V₇ and V₂₁; in addition, saturation calculations showed that green rusts should not be formed. In contrast, Mössbauer data showed total Fe in solids after 21 d of anaerobic incubation included about 6 % of paramagnetic FeII like green rust (that nearly totally disappeared during the 2 days of aeration), 11 % of paramagnetic FeII in minerals like sulfides (that did not change during the 2 days of aeration), and about 13 % of magnetic FeII/FeIII in minerals (that only partly

evolved during the 2 days of aeration, since there are still 11 % of Fe as magnetic FeII/FeIII in minerals at the end of this incubation). The differences between solubility calculations and Mössbauer measurements may result from (i) thermodynamic calculations that only tell us about the possibility of mineral formation and the solubility constants that vary for minerals having colloidal dimensions, and (iii) the choice of Lorentzian profiles to analyse experimental spectra privileging the representation of all types of FeII environments rather than actual FeII minerals (e.g. additional analyses non shown were performed using the Lorentzian profile for siderite and showed that a few percents of total solid Fe could exist in this form).

The return to aerated conditions corresponds simultaneously to a sudden change in O₂ status, and in CO₂ partial pressure that decreased and should have induced an initial increase in the pH of the slurry, although the oxidation of FeII could have thereafter induced a higher decrease in pH. These changes suggest the possible dissolution of Fe carbonate precipitates from the very start of the aeration, and of all FeII minerals as soon as FeII oxidation in solution has sufficiently decreased FeII concentration in solution. Under oxic conditions, [Duckworth and Martin \(2004\)](#) reported dissolution rate of FeCO₃ below 10⁻¹⁰ mol m⁻² s⁻¹ for 6.0 < pH < 10.3. These low values may indicate that the dissolution of siderite was not important in our incubation, all the more the initial FeII dissolution could have been comprised between about 7 10⁻⁶ mol.m⁻².s⁻¹ for FeII minerals having a diameter about 1 µm to 3.6 10⁻⁵ mol.m⁻².s⁻¹ for FeII minerals having a diameter about 2.5 µm.

In contrast to FeII oxidation, the literature indicated that MnII oxidation is mainly a biologic reaction. Biotic MnII-oxidation is generally led by microorganisms that can mediate biotic FeII-oxidation. However in the case of MnII-oxidizing bacteria all grow and oxidize MnII around neutral pH ([Tebo and He, 1999](#)). In [Davies and Morgan \(1989\)](#) experiments, MnII abiotic oxidation was performed with an oxidation constant *k* lower than 3.88 10⁻⁸ s⁻¹ at pH 8.35 or equal to 1.50 10⁻⁶ s⁻¹ at pH 8.95, whereas microbial MnII oxidation was around 2.90 10⁻³ s⁻¹ at a pH of 7.2 in experiments of [Katsoyiannis and Zouboulis \(2004\)](#). Under these conditions, abiotic MnII oxidation was low and does not proceed in practice ([Morgan, 2005](#)). The half life of abiotic MnII oxidation would be 350 d at pH 8 by abiotic oxidation (oxidation constant = 2.31 10⁻⁸ s⁻¹ ([von Langen et al., 1997](#))), whereas half life of microbial MnII oxidation would be 4 min (at pH 7.2, MnII oxidation constant = 2.90 10⁻³ s⁻¹). Moreover, [Morgan \(2005\)](#) reported half lives of MnII oxidation at pH 8 of 10 h for bacterial oxidation and 400 d for homogeneous solution oxidation. Then in our experiments, microbial

MnII oxidation did not proceed, and abiotic oxidation was too low to be observed into 2 d of aerobiosis.

Saturation calculations in soil solution indicated possible precipitation of MnCO_3 at the end of the anaerobic incubations of the 2 treatments V_2 , V_7 and V_{21} . After the switch to the aerobic incubation, saturation calculations suggested possible dissolution of these carbonate precipitates, concomitantly to the decrease of CO_2 to 0.04 % and pH increase. Duckworth and Martin (2004) observed formation of MnIII/IV hydroxides (bixbyite, birnessite, pyrolusite, nsutite) after dissolution of MnCO_3 . For $\text{pH} < 7.7$, MnCO_3 dissolution was low, and the rate constant for $\text{pH} > 7.7$ was about $10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}$. In their study, dissolution of MnCO_3 , led to bixbyite for $5.8 < \text{pH} < 7$ and feitnkechtite for $\text{pH} > 7.7$. In our experiments, MnS was near equilibrium saturation state after 21 d of anaerobiosis.

Anaerobiosis favours the production and/or the accumulation of mineral (HCO_3^- , CO_3^{2-} , NH_3 , HS^- , S^{2-}) and organic ligands (organic acids, amino acids) that can complex FeII and MnII, and increase their mobilisation (Millero et al., 1987) – the metal contained in the complexes being slowly oxidised with regard to free cations in solution (Urrutia et al., 1999) –. However, at the end of the anoxic period for the 3 treatments, FeII and MnII were mainly as free cations in solution, i.e. up to 81 % for FeII (about 15-19 % of FeII was complexed with organic acids) and 65 % for MnII (complexation with organic acids ranged between 12-18 %). At the end of the V_{21} anaerobic incubation, 17.5 % of MnII in solution were complexed with CO_3^{2-} . For all treatments, SO_4^{2-} complexation represented less than 2 % of FeII in solution and 4.5-2 % of MnII. These results suggested that the fate of organic acid after the return to aerobic conditions would have a minor effect on FeII and MnII mobilisation, the decrease in CO_2 partial pressure after 21 d of anaerobiosis could have slightly reduced mobilised MnII as we observed (Fig. 4a), and SO_4^{2-} fate can be neglected in a concern of FeII and MnII complexations. Other ligands did not significantly contribute to FeII and MnII complexations and mobilisations (NH_3 , SO_4^{2-} ...).

V.4.2 Microbial activities and dynamics after the return to aerobic conditions

Under aerobic conditions, organic compounds and NH_3 oxidations were a priori only microbial ones (van Bodegom et al., 2001), other ones such a sulphide oxidation could result

from simultaneous microbial and abiotic contributions (Edwards et al., 2000). With regard to the evolution of all these compounds, aerobic microbial activities seemed to be low with regard to current levels. More precisely:

- carbohydrates, including small ones, were not totally consumed in the anaerobic incubation of treatment V₂, and the degradation of the small ones carried out during the aerobic phase. However, their mean degradation rate of the 2 aerobic days period was only $3.50 \cdot 10^{-8} \text{ mol C l}^{-1} \text{ s}^{-1}$, inferior to values in literature ($2.4 \cdot 10^{-6}$ – $7.2 \cdot 10^{-6} \text{ mol C l}^{-1} \text{ s}^{-1}$) (Beltrame et al., 2004). In V₇ and V₂₁ treatments, all small sugars were consumed during the initial anaerobic incubation, the required initial hydrolysis of other carbohydrates remaining in solution being generally the limiting step in their microbial consumption (Glissmann and Conrad, 2002). During the following aerobic period for these 2 treatments, we noted a slight decrease of carbohydrates ($2.39 \cdot 10^{-9} \text{ mol C l}^{-1} \text{ s}^{-1}$) for treatment V₇, and an increase in small sugars and total carbohydrates for treatment V₂₁. These variations probably result from low microbial consumption and microbial excretion in treatment V₂₁ that could have resulted from a possible stress of microorganisms or death;
- Organic acid concentrations were stable during the 2 d of aerobic incubations, whatever the treatment, at the exception of butyrate that slightly decreased;
- SO₄²⁻ fate depended on the treatment: in V₂ and V₇ treatments, a slight increase of SO₄²⁻ the first 0.5 h of aerobiosis, may be attributed (i) either to partial sulphide re-oxidation into SO₄²⁻, (ii) either to processes of desorption due to pH and finally (iii) either to cells excretions
- Re-oxidation of NH₄⁺, as we could expect due to pH decrease, may have happen just for the first 0.5 h of V₇ and V₂₁ aerobic phase. Then NH₄⁺ in solution increase may be due to bacterial mortality. Increase of carbohydrates after aerobic switch in V₂₁ treatment may confirm bacterial excretions due to possible death of stress.

MnII concentration in solution (in the range of $3\text{-}5 \cdot 10^{-3} \text{ mol l}^{-1}$) should have had toxic impact on microbial activities and dynamics, as reported in literature (Adams and Ghiorse, 1985, Brynhildsen and Rosswall, 1997). Adams and Ghiorse (1985) showed a dramatic decrease of microbial growth and cellular yield for $9.1 \cdot 10^{-4} \text{ mol l}^{-1}$ MnII concentration. Addition of $1.8 \cdot 10^{-3} \text{ mol l}^{-1}$ MnII solution to a soil led to fall in of growth of *Leptothrix discophora* during the 80 h after addition, following by a very low growth ($2.22 \cdot 10^{-5} \text{ s}^{-1}$). In addition, the small observed microbial activities could have resulted from the death of several aerobes during the

initial anaerobic incubations, and the death of obligatory anaerobes after the return to aerobic conditions. Indeed, the initial anaerobic phase has favoured the growth of anaerobes, including a large proportion of fermentative bacteria that could be strict anaerobes (e.g. *Clostridium* spp.) (Dassonville et al., 2004). However, during the past 30 years, it has turned out that bacteria, previously considered as strict anaerobes, were able to cope with O₂ (Carter et al., 1995; Cypionka, 2000; Fareleira et al., 2003; Jean et al., 2004). As aerobic and facultative bacteria, many obligate anaerobes possess appropriate mechanisms against the O radicals and can only tolerate to some extent low levels of O₂ (Rolfe et al., 1978; Morris 1979). These findings reveal that the obligate anaerobes differ in their sensitivity to O₂, varying from those showing a strict intolerance to others possessing some intrinsic tolerance. The main hypotheses for the protective mechanisms are the ability to produce superoxide dismutase (SOD) and catalase to counterbalance the cell damage caused by O₂-derived toxic radicals. Sulfate-reducing bacteria, classified as obligate anaerobes, show an apparent ability to survive to oxygen exposure by the production of protective enzymes (Hatchikian et al., 1977; LeGall and Xavier, 1996; Cypionka, 2000). Despite the ability of *Desulfovibrio* species to cope with O₂, sustainable growth of pure cultures in the presence of O₂ has never been unequivocally demonstrated (Cypionka, 2000). However in Fareleira et al. (2003) experiments, *Desulfovibrio gigas* cultures survived to 24 h exposure to O₂ and resumed growth when shifted back to O₂. Patureau et al (1996, 2000) isolated a wide variety of denitrifiers from ecosystem adapted to fluctuating oxic-anoxic conditions. Bacteria exhibited aerobic denitrifying activity, i.e., co-respiration of O₂ and NO₃⁻ and simultaneous production of NO₂⁻ or N₂ gas (Patureau et al., 2000; Carter et al., 1995). *Clostridium perfringens*, considered as strictly anaerobic bacterium, can also survive when exposed to O₂ for short periods of time in a growth-arrested state (Trinh et al., 2000; Briolat and Reysset, 2002).

In spite of that, we observed changes in biodiversity – that had already been modified during the anaerobic first incubation (Fig. 3) – during the second aerobic period (e.g. for treatment V₇ in Fig. 8).

Les analyses ultérieures des spectres PCR-SSCP, nous renseigneront sur la dominance de certaines populations au cours de l'anaérobiose, comme observé par Dassonville et al. (2004) et si possible les évolutions de la biodiversité microbienne, des aérobies et anaérobies au cours du passage d'anaérobiose en aérobie.

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VI Discussion et conclusion

VI.1 Introduction

L'objectif de ce travail était de contribuer à l'évaluation des risques de mobilisation d'éléments métalliques préexistants dans les sols (Fe, Mn ...) suite à un apport de déchets liquides chargés en composés fermentescibles. Cet objectif correspond à une demande concrète de différentes institutions (ADEME, CIRAD, INRA, Chambre d'Agriculture de La Réunion).

Répondre totalement à cet objectif supposerait de comprendre et savoir décrire :

- les variations de l'état d'aération du milieu (et de conditions redox) avec l'apport de déchets (extension spatiale et variation temporelle) ;
- les processus de mobilisation (ou d'immobilisation) d'éléments métalliques en condition réductrice, en tenant compte de variations conjointes d'autres caractéristiques du milieu (acidité, capacité de complexation ...) ;
- la persistance sous forme mobilisée des métaux avec le passage à des conditions oxydantes (par transfert ou par aération du milieu) ; en définitive, leurs éventuels impacts en terme de biodisponibilité ou toxicité pour les plantes ou la microflore, ou de transfert et contamination des ressources en eaux souterraines ou de surface.

Dans le cadre de ce travail de thèse, notre contribution aura plus spécifiquement porté sur la réactivité biogéochimique du milieu avec comme objectifs scientifiques de :

- 1) hiérarchiser les besoins de description des grandes voies du catabolisme anaérobie des composés organiques en fonction de la nature de ces derniers pour définir leur contribution à la mobilisation des métaux ;
- 2) étudier plus spécifiquement la réduction de FeIII et de MnIV en anaérobiose et leur interdépendance avec les autres transformations biogéochimiques : (i) les contributions relatives des réductions biologiques directes et abiotiques à ces réductions, (ii) pour les réductions biologiques directes, l'impact de différents types de donneurs d'électron (H_2 , acides organiques, sucres) et accepteur d'électrons (NO_3^- ,

SO₄²⁻), et (iii) la physiologie des réducteurs de FeIII (aptitude à croître sans réduction de FeIII ou à effectuer d'autres catabolismes (dénitrification, sulfato-réduction, fermentations, ...)) ;

- 3) évaluer la persistance en solution des éléments métalliques mobilisés suite à leur transfert vers des zones oxygénées, ou à une amélioration de l'aération du milieu, ainsi que la résilience des effets de l'anaérobiose sur la composition minéralogique et la microflore.

Le travail a été réalisé sur un Ferralsol amendé avec une vinasse de rhumerie sous incubation en batch. La généralité des résultats a été recherchée au travers la caractérisation et la quantification des processus dans une optique de modélisation mécaniste ultérieure : il s'agit de faciliter la transposition des résultats à d'autres situations (types de sol, produits épandus), voire à d'autres problématiques liées à la présence de conditions anoxiques comme les émissions de gaz à effet de serre (N₂O, CH₄), l'immobilisation de radionucléides, la dépollution des sols en certains hydrocarbures ou en NO₃⁻ ...).

VI.2 Acquis de la thèse

VI.2.1 Voies majeures du catabolisme anaérobie

Dans un sol soumis à un apport d'une vinasse de rhumerie (sucres, polyols, acides organiques, alcools, protides, phénols ...) en conditions anaérobies, la dégradation des polyols, glucides, protides et acides organiques est simultanée. Elle peut être décrite au travers de quelques grandes voies cataboliques sans qu'il soit nécessaire de tenir compte de la spécificité des composés appartenant à chacune de ces familles. Liées à la nature de nos apports, les fermentations, suivies de l'acétogénèse réductrice de protons, étaient largement prépondérantes en début d'incubation. A l'inverse, la réduction de NO₃⁻ par dénitrification ou réduction dissimilative en NH₄⁺ était négligeable en terme de consommation de matières organiques, voire du H₂ produit au cours des fermentations ou de l'acétogénèse réductrice de protons. De manière similaire la réduction du SO₄²⁻ pouvait consommer 10-20 % du H₂ produit ou induire un défaut de production de H₂ équivalent. Au contraire des expériences de Dassonville (2003), la méthanogénèse est apparue après 21 j d'incubation par voie acétoclaste exclusivement – donc sans consommation de H₂ – après la fin des fermentations et de

l'acétogénèse. L'homo-acétogénèse fut *a priori* absente ou peu importante au vu des quantités de CO₂ produites, comparables à celles attendues parallèlement aux produits organiques des fermentations.

Les voies fermentaires et l'acétogénèse réductrice de protons semblent expliquer à elles seules la totalité de la consommation des composés organiques, apportés avec la vinasse, au cours des 21 premiers jours. Cela nous amènent à supposer que la réduction de FeIII et MnIV se fait principalement avec l'oxydation concomitante de H₂ produit au cours de ces fermentations et de l'acétogénèse réductrice de protons comme le suggéraient Lovley et al (1989), Coppi et al. (2004) et Dassonville et al. (2004). Dans nos expériences, cette hypothèse apparaît être d'autant plus crédible que la mobilisation de Fe et Mn diminue au-delà de 21 j, date aux environs de laquelle la production de H₂ a due cesser avec l'arrêt des fermentations et de l'acétogénèse réductrice de protons. Par ailleurs, au cours des expériences d'ajout de composés organiques, il a été constaté un faible effet de l'ajout de donneurs d'électron sans doute lié au maintien de certains composés apportés antérieurement avec la vinasse, à l'exception de H₂.

VI.2.2 Réduction et mobilisation des métaux

L'apport de vinasse et la mise en place simultanée de conditions anaérobies ont provoqué une mobilisation des métaux au cours des 21 premiers jours d'incubation en anaérobiose, aboutissant à des concentrations respectivement de $4 \cdot 10^{-3}$ à $7.7 \cdot 10^{-3} \text{ mol l}^{-1}$ et $3.9 \cdot 10^{-3}$ à $5.31 \cdot 10^{-3} \text{ mol l}^{-1}$ pour FeII et MnII. La mobilisation a débuté immédiatement pour MnII tandis qu'une phase initiale de latence d'une journée environ a été observée pour la mobilisation du FeIII.

Deux méthodes indirectes nous ont permis de vérifier que la majeure partie du Fe et du Mn réduit était en fait immobilisée. La première méthode supposait que le H₂ produit fut exclusivement consommé par la réduction du Fe et du Mn, ainsi qu'à la réduction de NO₃⁻ et de SO₄²⁻. Environ 15% de FeII et MnII en solution ont ainsi été réduit sans qu'il soit en fait possible de distinguer les 2 éléments. La deuxième méthode était basée sur l'analyse des cinétiques de mobilisation de FeII et MnII : elle aboutit à des estimations de FeII et MnII en solution de l'ordre de 15 – 30 % du FeII et du MnII totaux produits. Les mesures directes de

FeII en phase solide (étape 2 & 3) par spectroscopie Mössbauer ont confirmé (voire accentué) la part relative de FeII en phase solide avec des estimations de l'ordre de 97 %.

La spéciation de FeII et MnII en solution indique que plus de 80 - 85 % de FeII et 65 - 70 % de MnII sont sous forme d'ions non complexés, les principaux complexant des 20 % restant étant l'acétate pour FeII et 15 % étant l'acétate et 15% de CO_3^{2-} pour MnII. Ainsi, la contribution des ligands organiques et minéraux à la mobilisation de FeII et MnII est-elle faible. La raréfaction de ces ligands, très abondants pour certains dans nos conditions expérimentales, suite à un changement de conditions du milieu ne pourra pas expliquer de grandes évolutions en phase liquide pour Fe ou Mn. Il n'en va pas de même pour certains éléments comme le Cr pour lequel les formes complexées sont prédominantes : ainsi 99 % avec le butyrate en début d'incubation, puis 99 % avec CO_3^{2-} après consommation de cet acide organique.

Sans nous avoir permis de quantifier la part relative des réductions microbiennes directes de FeIII et MnIV par rapport à leur réduction microbienne indirecte (la réduction proprement dite de FeIII et MnIV étant alors abiotique), les expériences de la 2^{ème} étape suggèrent une forte contribution microbienne indirecte (i.e. avec intervention abiotique) à la réduction de Fe et Mn. Ainsi, l'accroissement en nombre des réducteurs de FeIII mesuré par mise en culture est-il toujours très faible par rapport à la création attendue de biomasse associée à la réduction de FeIII (moins de 0.01 %). De plus, la dynamique des réducteurs de FeIII ne semble pas toujours en accord avec la réduction ou la mobilisation observée de FeII.

Nos expériences nous ont permis de vérifier que les réducteurs de FeIII sont aussi aptes à d'autres fonctionnements cataboliques. Tous les réducteurs de FeIII étaient sulfatoréducteurs, alors que moins de 10 % étaient réducteurs du NO_3^- ou fermentaires, exception faite des résultats spécifiques à quelques traitements relatifs à l'ajout de glucose ou de lactate. Par conséquent, leur dynamique ne dépend pas exclusivement de la réduction des métaux mais également de l'évolution dans le temps de leur biodiversité fonctionnelle en lien avec l'expression de ces autres fonctions.

L'apport de NO_3^- conduit à la décroissance, voire la disparition, des bactéries réductrices du FeIII, et des quantités de FeII dans le système. Ces phénomènes peuvent être attribués d'une part à un effet toxique sur les réducteurs de FeIII de NO_2^- produit lors de la réduction du

NO_3^- , d'une part, et aux réactions abiotiques entre NO_3^- et FeII en solution et/ou en phase solide, d'autre part. Ces réactions abiotiques n'ont été mises en évidence auparavant que pour des interactions entre rouille verte sulfaté et NO_3^- (Hansen et al, 1996).

VI.2.3 Oxydation et immobilisation des métaux suite à un retour en aérobose

En accord avec la littérature, l'immobilisation de FeII en solution fut rapide en condition aérée, de 3 h à 1 j dans ce travail. Cette immobilisation serait la résultante de 3 processus : (i) l'oxydation de FeII en solution en FeIII, (ii) la précipitation de FeIII, et (iii) la dissolution progressive de minéraux contenant du FeII et néoformé durant la phase précédente d'incubation sous anaérobiose. L'oxydation en solution serait essentiellement abiotique, au vu de la rapidité du phénomène et des possibilités d'oxydation abiotique rapide bien connues pour cet élément pour des pH supérieurs à 5 (Davies et Morgan, 1989). La durée d'immobilisation de FeII est d'autant plus longue que l'élément est abondant en phase solide ; la disparition de FeII de la solution serait en partie contrebalancée par la dissolution de minéraux contenant du FeII, mais cette dissolution serait suffisamment lente ou serait très lente pour une partie de ces minéraux permettant ainsi la disparition presque totale de FeII en solution sans disparition totale des minéraux contenant du FeII néoformés lors de l'incubation anaérobie. La confrontation entre nos expériences et un modèle sommaire ne décrivant que l'oxydation en solution de FeII et la dissolution d'un minéral hypothétique contenant du FeII semble confirmer cette interprétation des processus.

Au contraire de FeII, MnII n'a pas disparu de la solution après les 2 jours d'aération des boues. L'oxydation abiotique de cet élément est un processus très lent, voire inexistant (Morgan, 2005) et les conditions de milieu n'ont pas permis la mise en place d'oxydation biologique. L'oxydation biologique de MnII en condition oxydante est mise en évidence dans les travaux de Katsoyannis et Zouboulis (2004) et Morgan (2005). Ainsi, la demi vie du MnII en solution à pH 8 serait de 10 h par oxydation microbienne et de 400 j dans le cas d'une oxydation abiotique.

Parallèlement, l'évolution des teneurs en ligands minéraux et organiques était très lente. Toutefois, FeII et MnII en solution étaient à plus de 65 % sous forme de cations libres et la complexation avec les acides organiques représentait moins de 20 % du FeII et MnII en

solution. Ainsi, l'évolution de leur concentration en solution n'aurait eu de toute façon que de faibles effets sur l'immobilisation de FeII par précipitation ou dissolution de minéraux contenant du FeII.

VI.3 Discussion sur les acquis de la thèse

VI.3.1 Considération sur la démarche expérimentale

VI.3.1.1 Expériences en batch

L'objectif de notre travail était d'aboutir à une bonne compréhension nécessaire à la hiérarchisation des processus affectant la mobilité des métaux (Fe, Mn) dans l'optique d'améliorer un modèle de réactivité biogéochimique à base mécaniste. Cet objectif nous a amené à nous placer délibérément dans des conditions extrêmes par rapport aux conditions réelles en matière de quantités de vinasse, de composés simples apportées, ou de durée d'anaérobiose. Cependant, les incubations en batch de boues de sol agitées, déjà utilisées par les équipes associées à ce travail, ont été retenues pour travailler sur un système homogène, effectuer des bilans de matière et faciliter ainsi l'identification des mécanismes. Cependant, ce dispositif expérimental amène à des artefacts non désirés tels que :

- (i) l'accumulation de CO₂ produit et ses effets sur le pH (Stumm et Morgan, 1996), la précipitation de carbonates (Pankow, 1991), et l'homo-acétogénèse (Diekert et Wohlfarth, 1994) notamment;
- (ii) l'accumulation de H₂ (faible dans nos travaux contrairement à ceux Dassonville (2003)) qui peut temporairement inhiber certaines réactions acétogènes vraies (Dolfing, 1988) et la méthanogénèse acétoclaste (Chin et Conrad, 1995) ;
- (iii) la mise en suspension et agitation des bactéries, susceptible de perturber les fonctionnements syntrophiques (McMahon et al., 2001).

Des alternatives de type système ouvert balayé en continu, ou en colonne « plug flow » fonctionnant en circuit fermé pourraient être plus appropriées, avec un apport faible et continu de nutriments (Feed batch). Ce dispositif proposé auparavant n'a pas été retenu pour des raisons de temps de mise au point.

VI.3.1.2 Dénombrements des bactéries par groupes fonctionnels ; estimation de la biomasse microbienne

Les dynamiques microbiennes de types fonctionnels ont été étudiées par dénombrement selon une méthode utilisant la mise en culture à différentes dilutions. Le choix des techniques de culture a été effectué en toute connaissance de ces imperfections :

- moins de 1 % des bactéries du sol sont cultivables sur les milieux de culture artificiels (Amann et al., 1995; Haveman et Pedersen, 2002) ;
- l'énumération effectuée est dépendante des milieux de cultures et de leur sélectivité plus ou moins restrictive (Vester and Ingvorsen, 1998).

Aussi, les comparaisons de dynamiques entre type fonctionnel devront être prudentes compte tenu des différences de cultivabilité entre groupes bactériens.

Bien que les avancées en microbiologie moléculaire (Hill et al., 2000) permettent de caractériser et d'étudier les groupes bactériens du sol à partir du 16S rRNA microbien directement extrait d'échantillons de sol et ultérieurement séquencés, la grande diversité phylogénétique et l'absence de gènes identifiés associé à chacune des communautés fonctionnelles rendent impossible l'énumération de bactéries sous groupes fonctionnels avec un gène spécifique (Loneragan et al., 1996).

Dans le cadre de ces expériences, nous avons tenté de suivre la dynamique de l'ensemble des micro-organismes par l'énumération de l'ensemble des anaérobies totaux sur un milieu de culture supposé adapté (Küsel et al., 1999). Il est évident qu'un tel milieu n'existe pas pour de nombreuses raisons incluant notamment les organismes syntrophes (Schink, 1997, Brown et al., 1999) ou l'effet de H₂ affectant certaines activités (Dolfing, 1988). Toutefois, le milieu retenu permettait de cultiver un spectre de bactéries beaucoup plus large que la somme des bactéries cultivables sur l'un ou l'autre des milieux de cultures spécifiques à une activité donnée (cf. chapitre IV). D'autres méthodes de dénombrement et/ou de mesure de la biomasse des anaérobies totaux auraient pu être envisagées. Ainsi, un marquage de l'ADN ou de l'ARN (acridine orange, DAPI) nous aurait-il renseignés sur la densité en nombre de micro-organismes vivants dans nos traitements et sur leur dynamique globale. Il n'aurait toutefois pas évité la prise en compte de micro-organismes aérobies stricts survivant au cours de nos épisodes anaérobies. Pour la 2^{ème} étape expérimentale, cette méthode de dénombrement total

sans mise en culture nous aurait renseignés sur la cultivabilité des anaérobies. Pour les expériences de la 3^{ème} étape expérimentale, elle aurait permis une première évaluation des conséquences d'un retour à l'aérobiose sur la résilience microbienne ; le couplage de ces données aux analyses PCR-SSCP déjà pratiqué par Dassonville et al. (2004b) nous aurait permis de passer à une interprétation semi-quantitative des spectres de PCR-SSCP. Cette méthode de dénombrement total se heurte toutefois à des difficultés liées au passage d'une densité en nombre à une biomasse, et au dénombrement simultané de cellules actives et inactives.

VI.3.2 Considération sur les résultats expérimentaux

VI.3.2.1 Production et consommation de H₂ ; la place des métaux

Les voies de consommation de H₂ autres que la réduction des métaux étaient la réduction de NO₃⁻ en NO₂⁻ (négligeable en terme de consommation de H₂) et la réduction de SO₄²⁻ (représentant une consommation ou défaut de production de 10-20 % d'H₂). Nous avons négligé les autres processus pouvant consommer H₂ en partie au vu des besoins en H₂ pour expliquer les quantités mesurées de FeIII et de MnIV réduits. Par ailleurs et plus spécifiquement à chaque type de biotransformation, (i) la méthanogénèse n'a été qu'acétoclaste et n'aurait débuté qu'après la disparition de H₂ du milieu, (ii) la fixation non-symbiotique de N ne paraissait pas s'imposer au vu des évolutions d'azote minéral, et (iii) l'homoacétogénèse ne semblait pas importante au vu des évolutions de CO₂, et notamment de la quasi-égalité entre les indices I_{fm} et I_{rf} sur des durées de 21 j. Dans la littérature sur la méthanogénèse, on avance des proportions respectivement inférieures à 33 % et supérieures à 67 % pour les voies hydrogénoclaste et acétoclaste (Fey et Conrad, 2000) en sol de rizière et l'on évoque des effets de l'agitation sur les méthanogène (Schink, 1997 ; McMahan et al., 2001). Concernant la réduction des métaux au cours des incubations après apport de substrats, H₂ semble avoir été un donneur d'électron privilégié pour la croissance des réducteurs de FeIII, suggérant qu'il ait été le donneur d'électron privilégié pour la réduction microbienne directe. L'apport de glucose a provoqué quant à lui une forte augmentation de la concentration en FeII en solution sans croissance significative des réducteurs du FeIII, suggérant une réduction microbienne indirecte de FeIII avec intervention d'autres communautés

fonctionnelles. Toutefois, il faut rester prudent dans ces interprétations en raison du caractère peut-être trop sélectif de notre milieu de culture pour les réducteurs de FeIII, et/ou de la possibilité de réduction de FeIII sans gain énergétique par des bactéries possédant d'autres fonctionnalités (Lovley, 1991).

Au vu des dénombrements des réducteurs du FeIII, les réductions indirectes du FeIII et du MnIV ont contribué significativement à la réduction du FeIII. Ces réactions abiotiques peuvent être couplées à la réoxydation de HS⁻ en S⁰ ou de S⁰ en SO₄²⁻. Les produits de réoxydation pourraient alors être à nouveau réduits par les bactéries sulfato-réductrices. A l'issue de nos expériences, nous n'avons pas pu déterminer les contributions respectives des réductions microbiennes et des réductions indirectes du FeIII. Ces estimations pourraient nous permettre d'affiner les relations entre la réduction du FeIII et la croissance des bactéries réductrices du FeIII.

VI.3.2.2 Distinction entre adsorptions/désorptions et absorptions/excrétions

Certaines observations nous ont amenés à considérer les phénomènes l'importance des phénomènes d'adsorption/désorption et/ou d'absorptions/excrétions des matières organiques provenant de la vinasse. Sans possibilité de distinguer strictement ces 2 familles de processus malgré les différences observées entre traitement stérile et traitement non stérile, nous avons supposé que les composés organiques vérifiaient une loi d'équilibre de type k_D entre solution et phases solides. Nous avons donc déterminé des coefficients de rétention basés sur les mesures en début d'incubation du sol avec la vinasse lors de la 1^{ère} étape expérimentale. Le bilan de C a ainsi été amélioré, en considérant cette rétention qui peut être négative (ainsi, on a observé une augmentation en arabinose, fructose et glucose en solution au-delà de ce qu'apportait la vinasse après un apport de vinasse ; par ailleurs, une augmentation des petits sucres lors du passage d'anaérobiose à aérobiose a été observée dans les expériences III). Cette approche mathématique reste très sommaire et pourrait être améliorée :

- d'une part en différenciant les processus d'adsorption/désorption physico-chimique et les processus d'absorption/excrétion microbienne ;
- d'autre part, en établissant pour les relations entre ces processus et les conditions de milieu : effet des variations des caractéristiques géochimiques du milieu (pH, le E_H, ...), ou des conditions nutritionnelles des micro-organismes

VI.4 Perspectives

VI.4.1 Compléments directs au travail de thèse

Trois domaines d'investigation pourraient donner lieu à travaux complémentaires plus ou moins ponctuels :

- en anaérobiose comme lors d'un retour à l'aérobiose, il serait important de pouvoir mieux caractériser l'évolution de Fe et Mn en phases solides (quantités réduites, nature des minéraux, aspects cinétiques des changements). Le passage de la technologie Mössbauer à la technologie EXAFS permettrait vraisemblablement de répondre à ces besoins ;
- la durée de 2 jours de la phase aérobie ultérieure à un épisode anaérobie s'est avérée trop courte pour appréhender les évolutions de MnII en solution, des acides organiques et de la biodiversité microbienne appréhendée par PCR-SSCP, et les évolutions des formes minérales de N et S. Il serait utile de reprendre ce travail en travaillant sur des retours à des conditions aérobies de plusieurs semaines ;
- un travail complémentaire pourrait être dédié à la caractérisation de contributions des adsorptions/désorptions et absorptions/excrétions de quelques composés organiques, en travaillant sur quelques molécules marquées et en confrontant traitements biologique et stérilisé.

VI.4.2 Extension du travail

Le modèle BIOGECHEM développé par [Dassonville et al., \(2004a\)](#) décrit les couplages entre microbiologie anaérobie et géochimie pour des sols où le catabolisme glucidique explique l'essentiel de la réactivité biologique. Dans sa version actuelle, le modèle décrit les dynamiques et activités de 5 types microbiens : dénitrifiants, fermentaires ($\times 2$), réducteurs de FeIII, acétogènes et sulfato-réducteurs, ainsi que les réactions géochimiques abiotiques en solution (sur la base d'équilibres thermodynamiques) et entre solution et phases solides (sur

des bases cinétiques) Au vu des travaux de [Dassonville et al. \(2004a, 2004b\)](#) et, plus généralement, des acquis scientifiques dans ce domaine, il apparaissait déjà nécessaire :

- d'ajouter certaines biotransformations comme la fixation non symbiotique de N_2 et la méthanogénèse ;
- de revoir la définition de certains types microbiens, notamment celle des réducteurs de FeIII qui n'avaient pas de voie catabolique alternative à la réduction de FeIII, alors que ces alternatives semblent être courantes ;
- de modifier les volets géochimiques abiotiques en conférant notamment un caractère cinétique aux réactions d'oxydoréductions (actuellement décrites sur la base d'équilibres thermodynamiques). Ces besoins supposent de remplacer le module figé de géochimie AQUA ([Vallès et Bourgeat, 1988](#)) actuellement utilisé dans BIOGECHEM par un modèle plus souple et couramment utilisé par la communauté internationale, à l'exemple de PhreeqC ([Parkhurst et Appelo, 1999](#)).

Nos travaux ont montré par ailleurs la nécessité d'autres modifications du modèle et nous permettent d'envisager certaines adaptations supplémentaires :

- l'extension du modèle à la présence de mélanges complexes de substrats par l'ajout de quelques réactions seulement. A noter que la description du métabolisme protidique peut rester sommaire dans le cas d'apport de vinasse car ces produits n'en sont pas trop chargés ;
- la prise en compte des phénomènes d'adsorption/désorption et absorption/excrétions de petits composés organiques qui ne pourra être effectuée qu'après acquisition de données complémentaires ;
- l'amélioration plus généralement de la description des processus impliqués dans la réduction et la mobilisation de Fe et Mn.

Enfin, si certains aspects des alternances entre anaérobiose et aérobie peuvent facilement donner lieu à modélisation (notamment les oxydations en solution, les précipitations des formes oxydées, et les dissolutions de minéraux solides contenant des formes réduites), d'autres questions cruciales restent actuellement sans réponses comme la survie des micro-organismes anaérobies (resp. aérobies) stricts en conditions aérobie (resp. anaérobies) et le maintien de leur potentiel pour certaines activités en condition défavorable, et les fonctionnalités majeures des organismes aérobies/anaérobies facultatifs. Derrière ce thème général se cachent des questions multiples plus ou moins spécifiques à certaines problématiques : besoins de distinctions de types microbiens en fonction de leur possibilités

de survie en conditions défavorables, maintien du niveau d'enzymes synthétisées dans des conditions très différentes de celles expérimentées ultérieurement par des micro-organismes
.....

Ces thèmes peuvent donner lieu à de nombreux travaux. Ainsi et à titre d'exemple, une suite est déjà envisagée sur l'étude des effets d'alternances répétées entre aérobose et anaérobose sur les micro-organismes aérobies/anaérobies et sur leurs fonctionnalités et ce, en fonction de différentes caractéristiques des périodes favorables et défavorables à leur développement (durée, niveau d'activité microbienne possible, toxicités ...). Au-delà de ces questions ayant trait à la résilience microbienne se posent aussi des questions relatives à la résilience chimique du milieu et notamment de ces phases solides facilement altérables.

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