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Molecular hydrogen from water radiolysis as an energy source for bacterial growth in a basin containing irradiating waste

Grégoire Galès [a,c,1], Marie-Françoise Libert [a,*], Régine Sellier [a], Laurent Cournac [b], Virginie Chapon [c], Thierry Heulin [c]

[a] *DTN/SMTM/LMTE, CEA Cadarache, F-13108 Saint-Paul-lez-Durance, France*
[b] *DSV/DEVM/LEP, UMR 6191 CNRS-CEA-Université Aix-Marseille II, CEA Cadarache, F-13108 Saint-Paul-lez-Durance, France*
[c] *DSV/DEVM/LEMIR, UMR 6191 CNRS-CEA-Université Aix-Marseille II, CEA Cadarache, F-13108 Saint-Paul-lez-Durance, France*

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Abstract

Although being deionized, filtered and therefore normally deeply oligotrophic, the water from a basin containing irradiating waste presented relatively high bacterial concentrations (ca 10^5 cfu ml^{-1}) and biofilm development at its surface and on the walls. This water was characterized by a high concentration of molecular H_2 due to water radiolysis, while its electrochemical potential was around +400 mV due the presence of dissolved O_2 and active oxygen compounds. This combination of H_2 availability and of an oxidant environment is completely original and not described in nature. From surface and wall biofilms, we enumerated the autotrophic populations ($\approx 10^5$ bacteria ml^{-1}) able to grow in presence of H_2 as energy source and CO_2 as carbon source, and we isolated the most abundant ones among cultivable bacteria. They efficiently grew on a mineral medium, in the presence of H_2, O_2 and CO_2, the presence of the three gases being indispensable. Two strains were selected and identified using their *rrs* gene sequence as *Ralstonia* sp. GGLH002 and *Burkholderia* sp. GGLH005. In pure culture and using isotope exchange between hydrogen and deuterium, we demonstrated that these strains are able to oxidize hydrogen as energy source, using oxygen as an electron acceptor, and to use carbon dioxide as carbon source. These chemoautotroph hydrogen-oxidizing bacteria probably represent the pioneer bacterial populations in this basin and could be primary producers in the bacterial community.

Keywords: Radiolysis; Hydrogen; Chemolithotrophy; Mass spectrometry; *Ralstonia*

1. Introduction

Due to their wide range of nutrients and energy sources, bacteria are able to colonize most natural and industrial environments. It is then not surprising when some bacterial biofilms occur in basins containing irradiating waste. Several microbial studies have been recently performed in nuclear spent fuel pools. These studies concerned essentially the formation of biofilms on submerged metal coupons [1–3]. In each case, the presence of biofilm-forming micro-organisms was observed in extreme conditions such as high radiation levels and low nutrient concentrations due to active water deionisation treatments. Heterotrophic micro-organisms have been found in bulk water of nuclear fuel storage basins [3]. They were able to transform, in experimental

* Corresponding author. Tel.: +33 44 2253144; fax: +33 44 2253545.
E-mail address: marie.libert@cea.fr (M.-F. Libert).
[1] Present address: UMR 6019 Dynamique des récifs et des plates-formes carbonatées Université de Provence 13003 Marseille cedex, France.

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conditions, esters, carbohydrates, and carboxylic acids. But what could be in situ the nutrient sources for bacterial development in such highly oligotrophic water?

In irradiating environments, radiations lead to the production of molecular hydrogen, hydrogen peroxide and some radicals ($OH^{\bullet}, O_2^{-\bullet}$) by radiolysis of water or embedding matrices [4–6].

In such environments, bacterial consumption of hydrogen has been demonstrated [7]. Bacterial oxidation of molecular H_2 commonly occurs in nature, as molecular hydrogen represents a high-energy reductant [8]. It is known that the ability of several bacteria to use hydrogen as an electron donor gives a substantial adaptative advantage in hydrogen-containing environments, like digestive track [9] or the rhizosphere of *Brassicaceae* sp. (Hartmann A. Institute of soil ecology, Neuherberg, Germany, personal communication). The use of hydrogen coming from groundwater radiolysis by bacterial strains isolated from groundwater has already been demonstrated [10,11].

Molecular hydrogen could then be one of the possible energy sources for bacterial growth in basins in which water radiolysis occurs. The aim of this study was to determine if autotrophic bacterial growth is possible in these basins. We investigated the presence of viable and cultivable micro-organisms by using cultivation media in heterotrophic conditions and autotrophic conditions supplemented with dissolved gas (H_2, O_2, CO_2, N_2). After isolation of autotrophic bacterial strains sampled from this environment, we focused our study on their hydrogen metabolism, characterizing their hydrogenase activity by mass spectrometry measurements.

2. Materials and methods

2.1. Basin water quality

The study was achieved in an environment containing irradiating waste submerged in demineralised water. The water was continuously treated by cooling and purification in a closed loop heat exchanger and a demineraliser. The radioactivity and chemistry of the water were analysed once a week. Concentrations of nitrate, nitrite, ammonium, sulphate and phosphate were measured by an ionic chromatography system equipped with conductivity detection. Dissolved oxygen and redox potential were measured with a multi-parameters analyser (Fisher Bioblock). Organic carbon was measured with a total organic carbon (TOC) analyser (Shimazu).

2.2. Water sample collection

Water samples were collected under sterile conditions, by submerging 50 ml sterile polypropylene tubes approximately 30 cm below the surface.

2.3. Isolation and cultivation of bacterial strains

2.3.1. Heterotrophic conditions

Counts of viable aerobic bacteria were achieved by plating on plate count agar (PCA) medium (Difco). Plates were incubated at 30 °C overnight. Colonies were then picked and purified several times by successive streaking. Sulphate-reducing bacteria were detected by using liquid medium according to a specific method previously described in [12].

2.3.2. Autotrophic conditions with dissolved gas as electron donor/acceptor

Autotrophic conditions were obtained in a liquid mineral medium (composition given in Table 1). Bottles of 100 ml were used, sealed with a rubber cap allowing continuous gas bubbling. Anaerobic conditions consisted in the use of the mineral medium with either nitrate or sulphate as electron acceptor, and a mixture of dissolved gases: H_2, 2%; CO_2, 1%; N_2, 97%. Aerobic conditions consisted in the use of mineral medium and a mixture of dissolved gases: H_2, 2%; air, 98%, or the mineral medium and a mixture of dissolved gases: H_2, 2%; O_2, 5%; CO_2, 1%; N_2, 92%. Counts of viable and cultivable bacteria were obtained with the most probable number (MPN) method. Isolation of colonies was performed by plating on mineral medium supplemented with agar (15 g l^{-1}), disposed in a sealed box allowing continuous gas bubbling and incubated at 30 °C until the bacterial growth was observed.

2.4. Bacterial identification

Dominant bacterial strains were identified by 16S rDNA sequencing according to previously described protocol [13,14]. Five microliters of a water-suspension colony were used for PCR amplification. The *rrs* genes (16S rDNA) were amplified using a pair of universal primers (*fD1* and *S6*) corresponding to positions 8–27 and 518–534, respectively, on the *Escherichia coli rrs* sequence [15]. The reaction mixture containing bacterial

Table 1
Composition of the mineral medium

Component	Concentration
KH_2PO_4	1.36 g l^{-1}
Na_2HPO_4	0.6 g l^{-1}
$MgSO_4 \cdot 7H_2O$	0.7 g l^{-1}
NH_4Cl	1.0 g l^{-1}
$CaCl_2$	0.1 g l^{-1}
$SO_4Fe \cdot 7H_2O$	0.02 g l^{-1}
$CuSO_4 \cdot 5H_2O$	5 μg l^{-1}
$H_3\ BO_3$	10 μg l^{-1}
$MnSO_4 \cdot H_2O$	10 μg l^{-1}
$ZnSO_4 \cdot 7H_2O$	10 μg l^{-1}
$Mo_7O_{24}\ (NH_4)_6 \cdot 4H_2O$	100 μg l^{-1}
$Co(NO_3)_2 \cdot 6H_2O$	10 μg l^{-1}
$NiSO_4 \cdot 6H_2O$	100 μg l^{-1}

cells was heated at 95 °C for 15 min before the addition of thermoresistant DNA polymerase (Eppendorf). PCR consisted of 25 cycles (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 65 °C for 2 min) according to the manufacturer. The final extension was at 65 °C for 3 min. The PCR product was purified using the QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed using the ABI PRISM Dye 10 Terminator Ready Reaction kit as specified by the manufacturer (Perkin–Elmer). Sequences were obtained with an automatic sequencer (ABI-PRISM 310 DNA-sequencer, Perkin–Elmer) using *S6* primer. The *rrs* sequences were first automatically and then manually aligned by comparison with a database of 35,000 already aligned bacterial *rrs* sequences.

2.5. Characterization of the hydrogen metabolism by measurement of hydrogenase activity

Pure bacterial strains obtained on agar plates under autotrophic conditions were grown on liquid autotrophic medium incubated with one of the gas mixtures cited above (H_2, 2%; O_2, 5%; CO_2, 1%; N_2, 92%). Because of their slow growth in autotrophic conditions, pure bacterial strains obtained on agar plates under autotrophic conditions were initially grown on liquid LB medium (l^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) and incubated with the precited gas mixture before testing their hydrogenase activity using an isotopic hydrogen–deuterium (H–D) exchange assay. After measuring it, we decided to make a pure culture of *Ralstonia* sp. GGLH002 in autotrophic conditions in order to test the effect of dissolved oxygen on hydrogen exchange. This culture was used in exponential growth phase at an optical density near 0.6 (600 nm).

The method used for the mass spectrometric measurements of gas exchange was previously described [16] and recently modified [17,18]. The cell suspension was placed in the measuring chamber (1.5 ml) of a mass spectrometer model MM 8-80, VG Instruments, Cheshire, UK. The bottom of the chamber (Hansatech electrode type) was sealed by a Teflon membrane allowing dissolved gases to be directly introduced through a vacuum line into the ion source of the mass spectrometer. The chamber was thermostated at 37 °C and the cell suspension was stirred continuously by a magnetic stirrer. The principle of the kinetic measurements of H_2 production, H_2 uptake, proton–deuterium (H–D) exchange, O_2 uptake or production, CO_2 uptake has been described previously [16,19]. The spectrometer sequentially scanned the abundance of the different gases (H_2, D_2, HD, O_2, CO_2) by automatically adjusting the magnet current to the corresponding mass peaks (m/e = 2, 4, 3, 32, 44, respectively). Measuring one mass peak typically took 0.5 s. Mass peaks were continuously recorded during the experiment.

The amperometric signal collected by the spectrometer is directly proportional to the gas concentration in the chamber, the proportionality coefficient varying from one mass to the other according to the ionization properties of the corresponding gas. In order to calculate the rates of gas exchange, the time derivation of gas concentrations has to be corrected from the slow but significant rate of gas consumption by the mass spectrometer, which superimposes to production or uptake rates. The mass spectrometer consumption of gases was assayed in cell-free buffer, it showed first order kinetics with time constants around 0.09 min^{-1} for H_2, 0.08 min^{-1} for D_2 and 0.024 min^{-1} for O_2; CO_2 consumption by the apparatus was negligible under the test conditions.

The set-up also allowed us to determine the in vivo hydrogenase activity by the use of hydrogen isotopes for the H–D exchange reaction in which D_2 disappears and is quantitatively replaced by HD and H_2. Isotopic exchange was measured by adding deuterium into the measuring chamber and closing the chamber just after reaching the solubility equilibrium. In case oxidizing equivalents are provided to the hydrogenase, H_2 uptake can be superimposed to the H–D exchange. According to [18], let us define $\Sigma = [HD] + [H_2] + [D_2]$ as the total concentration of dihydrogen species, $d\Sigma/dt$ the net uptake of H_2 ($d\Sigma/dt < 0$), V_{exch} the velocity of the H–D exchange reaction (i.e. the turnover rate of hydrogen species cleavage and recombination with water protons at the hydrogenase active site); and $\tau = ([D_2] + 0.5[HD])/\Sigma$ the isotopic ratio of D in hydrogen. When a simple isotopic exchange occurs ($d\Sigma/dt = 0$), the hydrogenase activity is computed from:

$$H_2\text{ase activity} = V_{exch} = 1/\tau(2 * d[H_2]/dt + d[HD]/dt).$$

When uptake occurs simultaneously with H–D exchange, we get:

$$V_{exch} = 1/\tau(2 * d[H_2]/dt + d[HD]/dt - 2(1 - \tau)d\Sigma/dt)$$

and

$$H_2\text{ase activity} = V_{exch} + |\, d\Sigma/dt \,| = V_{exch} - d\Sigma/dt$$

(see [19] for details).

3. Results

3.1. Ambient parameters

Physico-chemical analysis indicated that the basin water was an oligotrophic oxygenated medium. TOC was about 12 mg l^{-1} at the surface and dissolved oxygen concentration was 7.0 mg l^{-1} (219 μM). Conductivity was 1.0 μS cm^{-1}. The temperature varied from 28 to 35 °C (mean daily values) depending on the seasons.

Table 2
Physico-chemical and microbiological parameters of the water

Parameter	Value
Temperature	30–37 °C
pH	5.5
E_h	400 mV (ENH)
Conductivity	1 μS cm^{-1}
Radioactivity	10,000 Bq l^{-1} (mainly ^{60}Co)
Sulphate concentration	18–22 μg l^{-1}
Nitrate concentration	15–40 μg l^{-1}
Phosphate concentration	0.6 μg l^{-1}
Molecular hydrogen concentration (computed)	About saturation
[O_2]	3.8–7 mg l^{-1}
[TOC] on surface	12 mg l^{-1}
[TOC] at 3 m depth	2.6 mg l^{-1}
[DOC] on surface	11 mg l^{-1}
[DOC] at 3 m depth	2.4 mg l^{-1}
[POC] on surface	1 mg l^{-1}
[POC] at 3 m depth	0.2 mg l^{-1}
CFU on surface	$10^5 \pm 0.5 \times 10^5$ cfu ml^{-1}
CFU at 3 m depth	6000 cfu ml^{-1}

The concentrations of nitrate, nitrite, ammonium, sulphate and phosphate in the bulk water were usually within a range of several μg l^{-1}. pH values varied from 4.7 to 6.0. Redox potential was approximately +400 mV (NHE) from 1.5 to 3 m below the surface. Due to special conditions and safety procedures, it was not possible to perform a direct in situ measurement of H_2 concentration in the basin, but this can be estimated: according to the thermal power of the basin (corresponding to the activity of stored material and to the power needed for basin temperature containment, which are known) and the H_2 yield for radiolysis in oxygenated water [20,21], the molecular hydrogen concentration should be above saturation quantities. Some uncertainty on hydrogen concentration estimation was due to our uncertainty on the dose absorbed by water. But the appearance of gas bubbles at the surface of the basin, the blue colour due to Tcherenkov effect and data from literature [22] comforted us in the hypothesis of high hydrogen concentration. The simultaneous presence of high concentrations of both H_2 and O_2 is unusual in nature, because hydrogen is generally found in anoxic zones. Physicochemical characteristics of the basin water are listed in Table 2.

3.2. Cultivable bacteria

Under heteterotrophic plating conditions, the average number of total viable and cultivable bacteria in the basin water was estimated at $1 \pm 0.5 \times 10^5$ cfu ml^{-1}, see Table 2, this population size represented an average calculated from several sampling points during a two year-analysis period. During the same period no cultivable sulphate-reducing bacteria was detected. Bacteria were isolated and purified on PCA medium, and then identified using *rrs* gene sequencing. They belonged to the genera *Ralstonia*, *Burkholderia*, *Micrococcus*, *Staphylococcus*, *Bacillus* and *Pseudomonas*. These results are in good agreement with those of Sarro et al. [1]. It is noteworthy that bacterial populations dramatically decreased with the depth (only 6.0×10^3 cfu ml^{-1} at 3 m depth) like the oxygen content did (4 mg l^{-1} at 3 m depth), see Table 2.

Under autotrophic and aerobic plating conditions (gas mixture containing: H_2, 2%; O_2, 5%; CO_2, 1.5%; N_2, 91.5%), the bacterial population size was estimated about 10^5 cfu ml^{-1} (i.e. about the same than in heterotrophic plating experiments, which indicates that autotrophic strains represent certainly the major part of total cultivable diversity). These values represented averages calculated as previously and with the same fluctuations. Colonies were selected, purified, and then identified using *rrs* gene sequencing on Petri dishes (agar: 15 g l^{-1}, mineral medium) flushed with the gas mixture containing: H_2, 2%; O_2, 5%; CO_2, 1.5%; N_2, 91.5%. They belonged to the genera: *Burkholderia, Ralstonia, Delftia* and *Acinetobacter*. Strains of *Burkholderia* and *Ralstonia* most probably represented the dominant populations among cultivable bacteria growing in the basin, because they were found in both conditions of culture (heterotrophic and autotrophic) and they far outnumbered (especially *Ralstonia*) the other bacterial populations. Two strains were selected from the populations of *Ralstonia* and *Burkholderia* coming from autotrophic cultures. The closest *rrs* gene sequence of strain GGLH002 *rrs* gene partial sequence was that of *Ralstonia pickettii* (strain 2000032023, *rrs* sequence accession number AY268176, 100% identity). This strain will be named *Ralstonia* sp. GGLH002. The closest *rrs* gene sequence of strain GGLH005 was that of *Burkholderia cepacia* strain LMG 14294 (sequence accession number AF097533) with 99.5% identity. This strain will be named *Burkholderia* sp. GGLH005.

Under autotrophic and strictly anaerobic conditions, no bacterial growth was observed. Also, under autotrophic and aerobic conditions, no growth was observed without H_2 addition. The presence of the three gases (H_2, O_2, CO_2) was required for growth in autotrophic conditions, indicating that O_2 should be the terminal electron acceptor for molecular hydrogen oxidation and that H_2 oxidation was necessary for CO_2 fixation.

3.3. Hydrogenase activity

The hydrogen metabolism of these bacteria was investigated by measuring their hydrogenase activity. We determined the capacity of *Burkholderia* sp. GGLH005 and *Ralstonia* sp. GGLH002 to catalyze the H^+/D_2 exchange reaction, i.e. to evolve HD and H_2 from D_2 and H^+, and measured their hydrogen uptake rates.

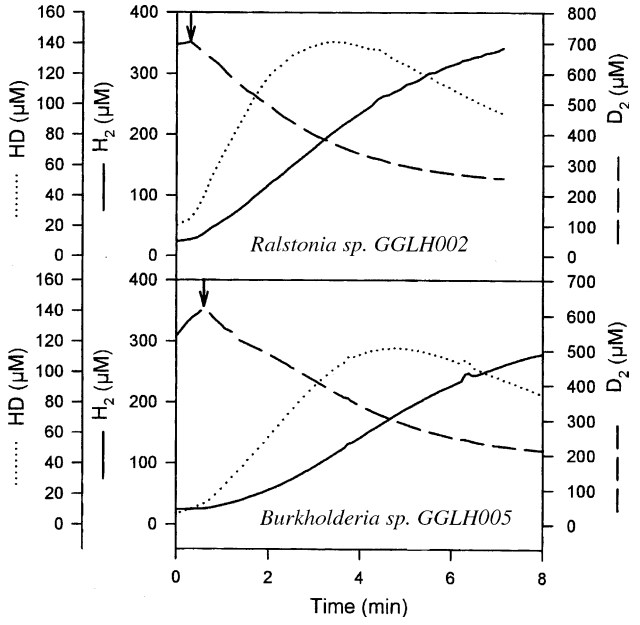

Fig. 1. Evidence of an hydrogenase activity in strains *Burkholderia* sp. GGLH005 and *Ralstonia* sp. GGLH002.

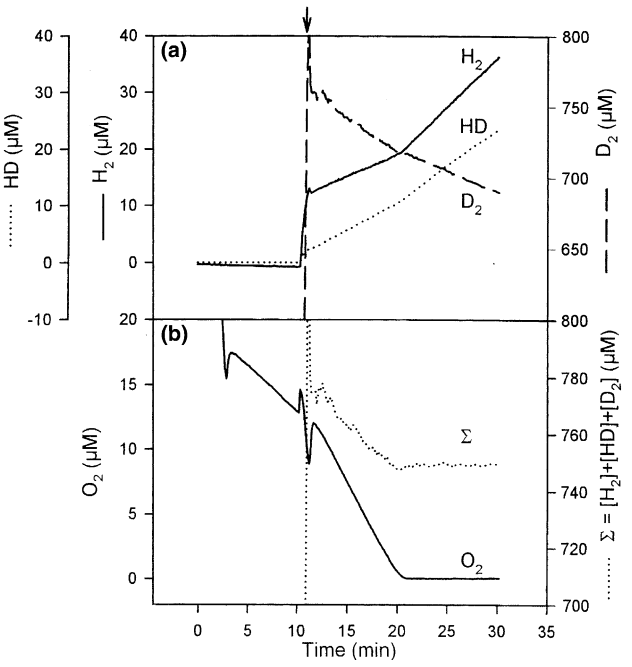

Fig. 2. Evidence of connections between hydrogenase activity and hydrogen/oxygen uptake in autotrophic conditions for *Ralstonia* sp. GGLH002.

Continuous recordings of the changes in concentration of dissolved gases from pure cultures of *Burkholderia* sp. GGLH005 and *Ralstonia* sp. GGLH002 grown in LB medium bubbled with the gas mixture (H_2, 2%; O_2, 5%; CO_2, 1.5%; N_2, 91.5%) are shown in Fig. 1. The bacterial culture (1.5 ml) was incubated at 37 °C in the MS-connected vessel and bubbled with D_2. In Fig. 1, the arrows represent the closure of the system after bubbling of D_2. The O_2 concentration rapidly decreased due to culture respiration. HD and H_2 concentrations rose immediately after the closure of the system. HD concentration decreased just 4 min later, as it acts as a transitory species, while H_2 concentration continued to increase. For both strains, hydrogen uptake was negligible in these conditions. Isotopic exchange seemed to be more efficient with *Ralstonia* sp. GGLH002 culture. It appears therefore that a significant hydrogenase activity occurred with both strains, but that hydrogen utilization was scarce in the measurement conditions (Table 3). As the culture became rapidly anoxic in the set-up, it can be hypothesized that hydrogen utilization was limited by the lack of O_2 as an electron acceptor.

Table 3
Isotopic exchange activity of two strains isolated from the basin in heterotrophic conditions

	Burkholderia sp. GGLH005 ($\mu M\ min^{-1}$)	*Ralstonia* sp. GGLH002 ($\mu M\ min^{-1}$)
Isotopic exchange (V_{exch})	200	250
H_2 uptake ($d\Sigma/dt$)	0	0
Hydrogenase activity ($V_{exch}-d\Sigma/dt$)	200	250

In order to test the potential connection between H_2, O_2 and CO_2 uptakes, gas exchange measurements were performed with an autotrophically grown culture. A pure culture of *Ralstonia* sp. GGLH002 was grown in mineral medium previously supplemented with the precited mixture of gases. Bacterial culture (1.5 ml) was incubated at 37 °C in the MS-connected vessel and bubbled with D_2. The D_2/H^+ isotopic exchange and the evolution of total H_2 concentrations in concern with O_2 and CO_2 concentrations were monitored (Fig. 2). Both graphs can be divided into three parts: in the first part, from 0 to 11 min, before bubbling of D_2, O_2 concentration decreased (0.7 $\mu M\ min^{-1}$) probably due to the aerobic respiration of *Ralstonia* sp. GGLH002. Then, from 11 to 20 min, after injection of D_2 and closure of the system, O_2 concentration decreased faster (1.5 $\mu M\ min^{-1}$) and there was a net uptake of H_2 (4.0 M min^{-1}). The isotopic exchange resulted in a production of HD and H_2 ($V_{exch} = 3\ \mu M\ min^{-1}$). The rate of production of these compounds was used to monitor the H–D exchange rate at the hydrogenase active site (see Section 2). Hydrogen uptake was assessed with the decrease of the total hydrogen content Σ (i.e. $[H_2 + HD + D_2]$). Total hydrogenase activity was the sum of H–D exchange and of hydrogen uptake and was equal to 7.0 $\mu M\ min^{-1}$. From 20 min to the end of the experiment, when the medium became anoxic, there was no more hydrogen uptake and the isotopic exchange rate increased (5.0 $\mu M\ min^{-1}$), total hydrogenase activity decreasing slightly. These results indicate that hydrogenase activity

Table 4
Connection of hydrogenase activity with hydrogen, oxygen and carbon dioxide uptakes for a *Ralstonia* sp. GGLH 002 culture in autotrophic conditions

	From beginning to 11 min (no D_2 injected) ($\mu M\ min^{-1}$)	From 11 min to 20 min (D_2 injected and oxic conditions) ($\mu M\ min^{-1}$)	From 20 min to the end (D_2 in the medium and anoxic conditions) ($\mu M\ min^{-1}$)
Isotopic exchange (V_{exch})	0	3	5
H_2 uptake ($d\Sigma/dt$)	0	−4	0
Hydrogenase activity ($V_{exch}-d\Sigma/dt$)	0	7	5
O_2 uptake	−0.7	−1.5	0
CO_2 uptake	Not measurable	−0.2	0

was not inhibited by O_2 in these conditions, and that O_2 was the main terminal electron acceptor for hydrogen utilization.

On other hand, CO_2 concentration was stable or slowly increased in the absence of hydrogen, while a CO_2 concentration decrease ($-0.2\ \mu M\ min^{-1}$) was observed during the period when both hydrogen and O_2 were present. Assuming that this strain realized the "knallgas reaction" (e.g. $2H_2 + O_2 = 2H_2O$), O_2 uptake could explain 75% of the hydrogen uptake. The other electron acceptor could be CO_2 which uptake was 0.2 $\mu M\ min^{-1}$; assuming that (at least) two molecules of H_2 were needed to reduce one molecule of CO_2, this could account for around 0.4 $\mu M\ min^{-1}$ hydrogen uptake. CO_2 uptake tended progressively to vanish in anoxia. This evolution of CO_2 concentration in regard with H_2 and O_2 uptakes suggests that CO_2 was an electron acceptor for the oxidation of H_2, but that CO_2 reduction needed respiration to be active. Results are summarized in Table 4.

4. Discussion

We investigated the microbiology of an unusual oxic environment containing dissolved hydrogen due to radiolysis of water, focusing on the putative autotrophy of hydrogen-oxidizing bacteria. The chemistry of such an environment was unusual, because the medium was oxidising whereas H_2 was present in high concentration. In natural environments, hydrogen is confined in anoxic zones. Hydrogen oxidation has already been described as an adaptative advantage in the colonization of the digestive track of mice [8,23]. Although the potential development of micro-organisms is thought to be very low due to the nutrient concentrations and to radioactivity, a total density of about 10^5 cfu per ml was found in the surface water. Due to safety procedures, we were not allowed to treat radioactive samples with classical dye of DNA under the microscope. Quantities of heterotrophically and autotrophically cultivable bacteria were similar, indicating that populations of cultivable bacteria were probably greatly dominated by autotrophic or mixotrophic species, as major cultivable bacteria like *Ralstonia* sp. GGLH002 and *Burkholderia* sp. GGLH005 were isolated from heterotrophic and autotrophic conditions. The autotrophic species *Ralstonia* sp. and *Burkholderia* sp. were identified as the major cultivable bacteria in the basin. Cultivable bacteria generally only represent a minor fraction of total bacteria [24]. A molecular study of bacterial diversity (16S rDNA cloning and sequencing) will demonstrate if both strains are really major strains in the basin (to be published). We showed that these strains grew with molecular H_2 as the sole source of energy and CO_2 as the sole carbon source. They are hydrogen-oxidizing bacteria using O_2 as the main terminal electron acceptor (as no H_2 uptake took place in anoxic conditions) and able to reduce CO_2 for growth as carbon source. They also grew on LB and LB 1/10 medium (data not shown), and could be very efficient scavengers of nutrients concentrated on the solid–liquid interfaces, making them particularly suitable for colonization of the entire ultra pure water system [25,26]. This metabolism seems to be a powerful adaptation to the ecological conditions prevailing in this basin.

The genera *Ralstonia* and *Burkholderia* could be characteristic of highly oligotrophic environments since they occur very commonly in ultra pure water industrial system [25,26]. Hydrogen concentration in the basin studied in this work were greater than the apparent K_m values determined for many other aerobic hydrogen bacteria [27], and is therefore certainly not a limiting factor for autotrophic growth of the hydrogenotrophic bacteria. Molecular hydrogen oxidation is probably an adaptative advantage that allows the bacterial colonization of the basin. It could be the basis of the ecosystem, allowing the further development of heterotrophic micro-organisms. Concentrations of other potential electron acceptors like nitrate, sulphate and CO_2 were far lower than those of dissolved O_2. Colonization seems to be more effective in the surface water, because there was a net decrease of the bacterial concentrations ($cfu\ ml^{-1}$) with regards to the depth, see Table 2. The air/water interface is the place where O_2 is more available and radiations less intense; this is also the main environment for biofilm development. Due to its high diffusion coefficient, H_2 concentration is likely homogeneous. Some hydrogen-based communities have already

been described [28]. There has been recently a controversy about the existence of hydrogen-based communities in subsurface environments, which were anaerobic [28–30]. Chapelle et al. [28] discovered an original hydrogen-based microbial community in deep subsurface, dominated by methanogenic Archaea. Bach and Edwards [31] found by the way of thermodynamical and geochemical arguments that hydrogen production in deep oceanic basalt aquifers could sustain the development of chemolithotrophic microbial communities. Wet storage of irradiating waste could then be an original environment containing aerobic hydrogen-based microbial ecosystems. Hydrogen oxidation by O_2 (Knallgas reaction) and nitrate reduction have indeed higher energy yields (per mole of electron acceptor) than iron reduction, sulphate reduction and methanogenesis. Oxygen is more available in this environment than nitrates. Molecular studies using culture-independent techniques should be used to thoroughly evaluate the abundances of the hydrogen-oxidizing and other bacterial genera in the basins, in order to validate this hypothesis.

Our work is the first report of a community probably based on aerobic hydrogen oxidation and also the first report of a community using hydrogen derived from the activity of irradiating waste as its main energetic resource. Further investigations are needed to better characterise this microbial ecosystem diversity and function, including a study of the molecular diversity of the bacteria using culture-independent techniques, as cultivable bacterial populations represent in general only a fraction of the total bacteria [24].

5. Conclusion

Previous microbiological studies in spent nuclear fuel storage basins in USA (Savannah river) or in Spain [1,3] indicated the presence of micro-organisms inhabiting such extreme chemical conditions, growing in biofilms and exhibiting a high density in the water column, but no explanation was available about the possible metabolic strategies of these micro-organisms. We demonstrated in this work that dissolved H_2 originating from water radiolysis could be the energy source for the bacterial development in the bulk water. Such a bacterial metabolism in this type of environment had not been suspected before.

Our data suggest that the first step of bacterial development in the basin could be represented by hydrogen-oxidising bacteria growing in the surface water, and then followed by the attachment of bacteria to metal surfaces and by microbial biofilm formation. This result is of importance for designing a proper biological management of nuclear storage basins. Analysis of bacterial communities inhabiting this environment will also certainly give opportunities to find strains with interesting properties, regarding their H_2 metabolism, and/or resistance towards oxygen and radiation.

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