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## Review

# Resource recovery from used water: The manufacturing abilities of hydrogen-oxidizing bacteria

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ABSTRACT

Resources in used water are at present mainly destroyed rather than reused. Recovered nutrients can serve as raw material for the sustainable production of high value bio-products. The concept of using hydrogen and oxygen, produced by green or off-peak energy by electrolysis, as well as the unique capability of autotrophic hydrogen oxidizing bacteria to upgrade nitrogen and minerals into valuable microbial biomass, is proposed. Both axenic and mixed microbial cultures can thus be of value to implement re-synthesis of recovered nutrients in biomolecules. This process can become a major line in the sustainable "water factory" of the future.



## Contents



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## 1. Introduction

The present approach to wastewater treatment is burdened by the heritage of the sanitary engineering: disintegrate the residual materials, make them disappear. In the conventional activated sludge (CAS) system, the wastewater is treated by means of an energy-demanding dissipative set of processes, aiming at the total decomposition of all organic molecules prior to return of the cleaned effluent to the ecosystem (Verstraete and Vlaeminck, 2011). Currently, wastewater is regarded as an assembly of resources to be recovered such as energy, nutrients (C, N, P etc.) and water itself. Each of them can be used as building blocks of all forms of life. The need to fully or partially revise the decomposing and dissipative aspects of the CAS system is currently addressed by scientific research, and this effort is nowadays attracting increasing attention (Hellström et al., 2008; McCarty et al., 2011; Mo and Zhang, 2013, 2012). The decrease of the environmental footprint, particularly by limiting greenhouse gas (GHG) emissions, and by focusing on the recovery of valuable resources, are urgent and mandatory issues in wastewater treatment systems (Daigger, 2009; Verstraete, 2002). When considering future perspectives towards innovative bio-treatments of anthropogenic waste streams, environmental biotechnology offers a virtually infinite set of bioprocess combination, which can theoretically lead to achieve remarkable results in terms of process innovation and efficiency. The latter and the developments in the field of renewable energies and process engineering, provide plenty of opportunities for serendipities (Mo and Zhang, 2013). In this framework, hydrogen-oxidizing bacteria can be considered as one of the most powerful microbial actuators of the transition towards integrated bio-refineries. They are ubiquitous bacteria with the ability to consume molecular hydrogen in their energy yielding process: it gives this group of microorganisms several nutritional advantages such as the ability to grow in an exclusively inorganic medium, converting rapidly $CO_2$ and reduced nitrogen into new cellular material (Ammann and Reed, 1966; Repaske and Mayer, 1976). Innovative approaches employing this kind of bacteria might be suitable for the upgrade of nutrients recovered from anaerobic digestate and reject waters in wastewater treatment plants (WWTP), as well as for carbon dioxide capture and upgrading in the process of converting biogas to biomethane. Implementing a circular approach, the basic components to be removed from liquid or gaseous streams would be not anymore "neutralized" but recovered and upgraded into new valuable microbial biomass rich in proteins, biopolymers or microbial oil. This mini-review aims to outline some of the main features and perspectives of hydrogen-oxidizing bacteria. It should be emphasized that these bacteria have been explored extensively almost half a century ago as potential working horses for microbial technology. The concept is that at this age of bio-economy, they can potentially find effective niches for useful application in the context of resource recovery from used water.

## 2. Hydrogen-oxidizing bacteria

Hydrogen-oxidizing bacteria or Knallgas bacteria (named after the gaseous mixture of $H_2$ and $O_2$ they consume) are aerobic, facultative autotrophic bacteria which share the ability to fix carbon dioxide into new cellular material by the ribulose biphosphate or reverse tricarboxilic cycle, using hydrogen and oxygen, respectively, as electron donor and electron acceptor in the energy yielding process (Khosravi-Darani et al., 2013). Indeed the change in Gibbs free energy and the resulting ATP formation at pH 7 (with a negative change in free energy) is substantial (Eqs. (1)–(3)) (Yu et al., 2013).

$$H_2(g) + 1/2O_2(g) \rightarrow H_2O(L) \quad \Delta G^0 = -237.1\ \text{kJ/mole} \tag{1}$$

$$ADP + Pi \rightarrow ATP \quad \Delta G^0 = +30.5\ \text{kJ/mole} \tag{2}$$

$$H_2(g) + 1/2O_2(g) + 7(ADP + Pi) \rightarrow H_2O + 7ATP \quad \Delta G^0 = -23.6\ \text{kJ/mole} \tag{3}$$

Besides possessing the key enzymes that allow them to grow with $H_2 + CO_2$ as the sole energy and carbon sources, these aerobic bacteria can support their growth also by oxidizing organic substrates such as sugars, organic acids and amino acids (Schink and Schlegel, 1978), therefore possessing mixotrophic metabolic capabilities. In their study on hydrogen metabolism in aerobic hydrogen-oxidizing bacteria, Schink and Schlegel (1978) identified them as a heterogeneous group of taxa such as *Alcaligenes*, *Pseudomonas*, *Paracoccus*, *Aquaspirilium*, *Flavobacterium*, *Corynebacterium* (Gram-negative genera) as well as *Nocardia*, *Mycobacterium and Bacillus* (Gram-positive genera). In general, they are all naturally occurring microorganisms, inhabiting niche environments where oxygen concentrations fluctuate around hypoxic conditions (oxic–anoxic threshold). In this way, they take advantage of the hydrogen released by anaerobic microorganisms without being affected by high $O_2$ concentrations.

### 2.1. Bio-products from hydrogen-oxidizing bacteria

Hydrogen-oxidizing bacteria are a special group of bacteria which attracted the attention of researchers already during 1970s, as potential producers of single cell protein (SCP) (Repaske and Mayer, 1976), biomass for fermentation industry and polyhydroxybutyrate (PHB) (Siegel and Ollis, 1984) (Fig. 1). Autotrophic cultivation of hydrogen-oxidizing microorganisms represents the core of many studies since this peculiar group of bacteria was discovered. The most representative and well-studied hydrogen-oxidizing bacterium is *Cupriavidus necator*, which name underwent several changes along the years: *Hydrogenomonas eutrophus*, *Alcaligenes eutropha*, *Wautersia eutropha* and *Ralstonia eutropha* (Khosravi-Darani et al., 2013). For reasons of clarity, we further on use most often the most recent name, i.e. *Cupriavidus necator*. The attention paid to this strain is due to its extremely flexible versatile metabolism, i.e. the capability of easily shifting between heterotrophic and autotrophic growth modes, using organic compounds or molecular $H_2$ as energy sources, both alternatively or concomitantly (Pohlmann et al., 2006). The research on bioprocesses related to this microorganism generated an interesting amount of information concerning its stoichiometry and kinetic parameters, which can be regarded as reference points for this group of bacteria.

### 2.2. Stoichiometry of hydrogen-oxidizing bacteria

The stoichiometry for autotrophic cell growth of *Cupriavidus necator* as indicated by Ishizaki and Tanaka (1990) is the following:

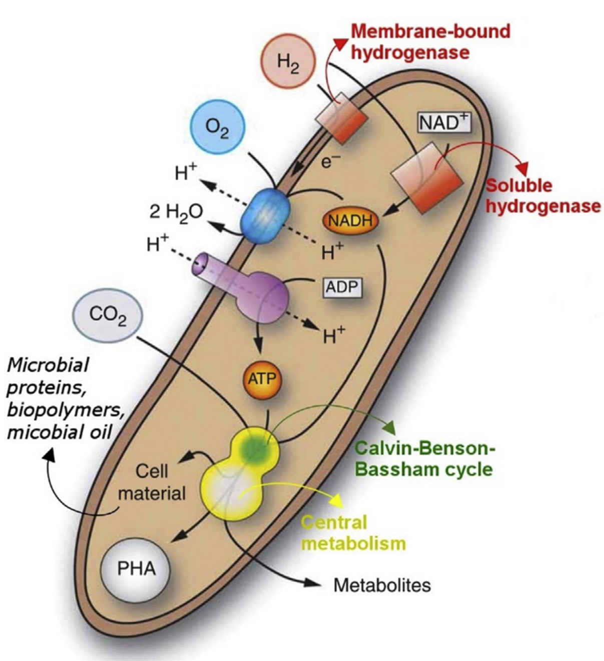


**Fig. 1 – Schematic representation of lithoautotrophic metabolism and bio-product formation in *Cupravidus necator*. Adapted from Pohlmann et al., 2006.**

$$21.36\ H_2 + 6.21\ O_2 + 4.09\ CO_2 + 0.76\ NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.70\ H_2O$$

The molar ratio of gaseous substrate consumption ($H_2/O_2/CO_2$) here reported, however, can change when other strains are considered, and depends on the growth conditions and the growth rate (Schink and Schlegel, 1978). The most common ratios of the gaseous substrate composition reported so far in different studies about hydrogen-oxidizing bacteria are of the order of $H_2/O_2/CO_2 = 7{:}1{:}1$ (*v/v*) (Tanaka et al., 2011) or 7:2:1 (*v/v*) (Volova et al., 2013). In this sense, an important metabolic parameter is represented by the $H_2$ uptake over $CO_2$ uptake ratio, which is regulated by the balance between the expenditure for catabolic energy and the level at which electrons enter the respiratory chain. $H_2/CO_2$ values ranging between 4 and 10 were reported as suitable for the growth of these microorganisms (Schink and Schlegel, 1978). Many studies have indicated the crucial role that the oxygen concentration plays in the metabolism of aerobic hydrogen-oxidizing bacteria. It is necessary as final electron acceptor, but inactivates the hydrogenase enzymes if present above certain limits (Vignais and Billoud, 2007). Oxygen inhibition of growth was indicated as strain dependent in earlier studies on *Cupriavidus necator* (Siegel and Ollis, 1984), with growth inhibition occurring already at $O_2$ concentrations of 4% (*v/v*), whereas recently the highly CO-tolerant *Ideonella* sp. O-1 was found as capable of growing in presence of $O_2$ levels greater than 30% (*v/v*) (Tanaka et al., 2011).

### 2.3. PHB: from bio-polymers to prebiotic

Amongst the interesting metabolic features of hydrogen-oxidizing bacteria, increasing attention has been paid to the accumulation of biopolymers, particularly by employing axenic cultures of *Cupriavidus necator*. Since earlier studies indicated how far developed this trait was in *Cupriavidus necator*, especially under oxygen limiting conditions (Siegel and Ollis, 1984), the autotrophic cultivation of this microorganisms for PHB production was studied by different groups of researchers (Volova et al., 2013). This process was regarded as a possible way of binding $CO_2$, coupling it to the production of biodegradable and renewable biopolymers (Tanaka et al., 1995; Volova et al., 2013). Recently, some studies highlighted also the possibility of using CO-tolerant hydrogen bacteria for PHB production on exhausted industrial emissions rich in $H_2$, $CO_2$ and CO (Tanaka et al., 2011; Volova et al., 2002). Besides the widespread research on PHB as raw material for bioplastic production, these bio-polymers have been recently shown to be able to act as microbial control agents when used in the diet of different aquaculture species (Najdegerami et al., 2012). Thus, PHB have also the potential to be used as anti-infective agents for aquaculture (Boon et al., 2013; De Schryver et al., 2010; Defoirdt et al., 2007), broadening the possible applications of this microbial byproduct.

### 2.4. Stoichiometry of PHB formation in hydrogen-oxidizing bacteria

Polyhydroxybutyrate (PHB), is a biopolymer used as energy storage by bacteria. They accumulate excess of carbon in form of biopolymers when low concentrations of other compound such as oxygen or nutrients limit their growth (Khosravi-Darani et al., 2013). The stoichiometry of PHB accumulation in *Cupriavidus necator* can be expressed as follows (Tanaka et al., 1995):

$$33\ H_2 + 12\ O_2 + 4\ CO_2 \rightarrow C_4H_6O_2 + 30\ H_2O$$

Hence, 1.30 kg of PHB could be theoretically harvested per kg of $H_2$ metabolized, which in energy terms corresponds to 0.16 kg PHB/kg $H_2$-COD. The remarkably high weight of PHB per kg $H_2$ is explained by to the fact hydrogen is the lightest existing molecule. On a COD basis, the PHB yield by hydrogen-oxidizing bacteria is comparable to other bacteria accumulating PHB from $C_1$ compounds (0.14 kg/kg methane-COD), whereas it is less favorable if carbohydrates (0.45 kg/kg glucose-COD), $C_2$ compounds (0.45 kg/kg acetic acid-COD) and $C_4$ compounds (0.64 kg/kg butyric acid-COD) are used as energy source (Yamane, 1993). This stoichiometry is assumed as representative also of other hydrogen-oxidizing bacteria, able to accumulate PHB under stress conditions.

### 2.5. *Axenic cultures, mixed microbial communities and microbiomes*

Amongst the numerous studies on hydrogen-oxidizing bacteria, there is, to the best of our knowledge, no report of lithotrophic mixed microbial cultures established on hydrogen, oxygen and carbon dioxide. Neither is there information to what extent such microbial cultures can evolve together and become gradually evolved and organized to achieve maximum growth efficiencies and yields. The microbial characterization of single bacterial strains able to oxidize molecular hydrogen in presence of oxygen allowed to obtain a vast knowledge about their physiology and metabolism (Schink and Schlegel, 1978) and their kinetic parameters (Siegel and Ollis, 1984). A major achievement in this line is the report on the genome sequencing of *Cupriavidus necator* H16 (Pohlmann et al., 2006). This allowed revealing microbial features that attracted the attention of researchers interested to explore the limits of specific bacterial strain under defined conditions. Nevertheless, the exploitation of axenic cultures in real scale applications faces problems of external contamination, and the measures to be taken often hamper scaling-up biological processes under such strictly sterile conditions. The exploitation of evolving microbial communities has one major disadvantage that is, generating a biomass whose composition cannot be assured to be constant. Yet, it offers several concrete advantages. In contrast to axenic cultures, a non-specific biomass is easy to acclimate to different environments, not requiring any strict sterile environment to carry out its metabolism (Chen and Gu, 2005a, 2005b). This more pragmatic approach is of vital importance when dealing with used water treatment and resource recovery. In this framework, when one considers a mixed microbial community adapted to a natural or artificial environmental niche, which has acquired a specific structure and metabolism, the term microbiome is appropriate (Marshall et al., 2013). Besides their ability to cope with rapid changes in environmental conditions (Allison and Martiny, 2008), an important feature of microbiomes is the ability to restructure themselves when subjected to a selective pressure. Therefore, by applying strict environmental niche conditions such as the supply of $H_2$, $O_2$ and $CO_2$, a generic microbial community can be rapidly enriched with specific hydrogen-oxidizing bacteria. Following the same line of development established for mixed cultures of methane-oxidizing bacteria and heterotrophic bacteria, autotrophic hydrogen-oxidizing bacteria might also take advantage of such autotrophic–heterotrophic interaction. A clear example of such advantage is reported for full-scale production of SCP from natural gas (Dalton, 2005), with the methane-oxidizing bacteria accumulating excess of acetate in the culture media. In that case, the shift from sterile to semi-sterile condition and the addition of heterotrophic bacteria allowed to remove by-products which were hampering the growth of autotrophic methane-oxidizing bacteria. This aspect has been recently elucidated also at lab scale: increased heterotrophic richness allowed to enhance the functionality of a methane-oxidizing bacteria in the same mixed microbial culture, with methane oxidation being the affected parameter (Ho et al., 2014). In the case of hydrogenotrophic bacteria, different interacting microorganisms might then become the backbone of a collaborating auto-heterotrophic microbiome able to use hydrogen and carbon dioxide and, moreover, ammonia with high efficiencies and without the need of strictly sterile conditions. The exploitation of such microbial community could allow for instance to recover ammonia from anaerobic digestate or reject water of municipal WWTP, upgrading all the simple components to valuable biomass rich in proteins and PHB.

## 3. Tackling the sustainability of feed production: microbial proteins from $H_2$, $CO_2$, $NH_3$ and $O_2$

The fixation of carbon dioxide into new cell material, generating new biomass and bypassing the photosynthetic pathway sun-plant-biomass, was the very first focus of early studies on hydrogen-oxidizing bacteria. The capability of these bacteria to rapidly grow in generic and inexpensive inorganic media ($m_{max} = 0.42\ h^{-1}$) (Ishizaki and Tanaka, 1990), achieving considerably high yields in terms of volumetric production rates (5.23 g CDW/L·h) (Tanaka et al., 1995), was investigated in several studies on the production single cell protein to be used as animal feed (Repaske and Mayer, 1976). Lepidi et al. (1990) made a first attempt to compare the energy efficiency of biomass production from hydrogen-oxidizing bacteria with the photosynthetic efficiency of the fastest growing plants. They proposed a process scheme where renewable energy was used for electrolysis of water, producing $H_2$ and $O_2$ to be used as gaseous substrates (together with $CO_2$) for biomass production in reactor systems. The estimated solar energy recovery of 2% as caloric power of microbial biomass was already higher than the photosynthetic efficiency of 0.5% of the fastest growing crop (Lewis and Nocera, 2006). Notably, the authors concluded that, based on their data several hundred tons of microbial biomass dry matter per ha per year could be produced in well-designed reactor systems (Lepidi et al., 1990). At present, the highest production levels of $C_4$ plants like e.g. maize are in the range of 10–20 tons dry matter per ha per year (Fischer and Edmeades, 2010; Grassini and Cassman, 2012); this is a fraction relative to potential that $H_2$-oxidizing bacteria may have per unit footprint when efficiently grown in reactor systems. Recently, a similar study employing an axenic culture of *Cupriavidus necator* in a closed reactor system estimated a solar energy recovery around 5% (Yu et al., 2013), and the overall efficiency of the solar hydrogen coupled to the microbial culture system was evaluated to be up to 10 times higher that of the conventional crop plants or microalgae. Besides being a sustainable and efficient alternative to photosynthetic biomass production, hydrogen-oxidizing bacteria can be regarded as a potential source of microbial protein, i.e. single cell protein (SCP) (Anupama and Ravindra, 2000). The suitability of hydrogen-oxidizing bacteria as SCP producers was recently investigated in a study on the characteristics of the proteins synthetized by these bacteria (Volova and Barashkov, 2010). The biological value of proteins synthetized by three hydrogen-oxidizing bacteria was assessed: *Alcaligenes eutrophus Z1*, *Ralstonia eutropha B5786* and the CO-resistant strain of carboxydobacterium *Seliberia carboxydohydrogena Z1062*. This study showed that the high content of protein synthetized by these bacteria possesses also has a complete profile of valuable amino acids. Indeed, the amino acids profile was similar to that of yeast, microalgae and casein, but the final content of protein of 70% (on a dry weight basis) for $H_2$-oxidizing bacteria was much higher than the respective values i.e. 50 and 15% measurable in yeast and wheat grain. Further, the total essential amino acids content of hydrogenotrophs was more than 10% higher than in grain and close to the content of casein. Moreover, the assimilation in the gastro-intestinal tract (simulated by availability for proteolytic enzymes *in vitro*) of such microbial proteins is about a factor 1.4 higher than that of wheat proteins and almost comparable to that of casein, which amounts to 44% for pepsin (after 3 h) and 55% for trypsin (after 6 h) (Volova and Barashkov, 2010). In view of the increasing scarcity of food proteins (land use limitations, water scarcity, climate change, increased demand (Hanjra and Qureshi, 2010; Lobell and Field, 2007; Tilman, 1999; Zepka et al., 2010)) and the long-term stability of augmented prices of feed and food on the world market (Supplementary Fig. 1-a,-b), it stems to reason that at present a renewed attention can be seen in the direction of developing microbiological technologies for protein synthesis on different gaseous and liquid substrates, particularly if the latter can be generated in a sustainable way (Nangul and Bhatia, 2013).

### 3.1. *New approaches to resource recovery: the $H_2$-based biorefinery*

The production of hydrogen from renewable energy sources is gradually replacing the generation from fossil fuels driven systems, and the technical advances in the energy sector are expected to lower the prices of green hydrogen production in the near future (Bartels et al., 2010). For instance, electrical energy efficiencies up to 73% are already achieved by commercial and industrial grade electrolysers, and researches on new materials and electrolysers configurations showed possible efficiencies as high as 96% (Mazloomi and Sulaiman, 2012). Hydrogen has always been regarded as a sustainable alternative to fossil fuels or as a mean of electrical energy storage (Coskun et al., 2011). Nevertheless, it can be also seen as a primary energy source in case of hydrogen-oxidizing bacteria. Hydrogen production costs from renewable energy sources vary accordingly to the considered scenario. Cost analyses available in literature suggest values of around 1.7 Euro/kg $H_2$ for natural gas reforming (which can be also applied to biogas) (Bartels et al., 2010), 1.2 Euro/kg $H_2$ for biomass gasification (Bartels et al., 2010) and 1.8 Euro/kg $H_2$ for hydrogen generation from wind energy (Gökçek, 2010). The latter was taken as reference to estimate the energetic costs related to the use of hydrogen-oxidizing bacteria, with a final reference value of 0.23 Euro/kg $H_2$-COD. The microbial performances of hydrogen-oxidizing microorganisms are resumed in the yields reported in Table 1, which calculations were based on stoichiometric data reported in literature about *Cupriavidus necator*. The yields were calculated on an equivalent $H_2$-Chemical Oxygen Demand ($H_2$-COD) basis. A comparison between the energetic costs related to hydrogen-oxidizing bacteria and the prices of similar products/processes already marketed is also reported in Table 1. As indicated, the production costs of microbial biomass rich in proteins are around two-fold the marketable price of soymeal. However, the average protein content of the latter product is of the order of 40% (Acikgoz et al., 2009), whereas hydrogen-oxidizing bacteria were reported as capable to accumulate as much as 75% of protein on a dry weight basis (Volova and Barashkov, 2010). These first considerations allow estimating a final raw protein cost comparable to the market price of soymeal used as reference protein feedstuff. Clearly, other opex and capex costs should not be neglected, but the latter are primarily related to the scale dimensions, and should certainly not triple the costs based on those of the primary ingredients i.e. hydrogen and oxygen. More realistic estimations of the final protein cost for raw materials needed in single cell protein production (Stanbury et al., 1995) indicates that an amount of 62% of total production costs is related to issues of utilities, labor and supervision, fixed charges, maintenance, etc. In this case, the marketable price of the single cell protein would be around 1.75 Euro/kg of protein, which is a factor 1.7 above the actual price of soymeal. Nevertheless, this estimated marketable price is still lower than e.g. the total production cost of 2.10 Euro/kg dry cell of yeast grown on molasses (Lee and Kyun Kim, 2001). When the production of biopolymers as PHB is taken into consideration, the energetic production cost is even lower than what is reported in literature for possible microbial PHB production on other substrates such as glucose and methanol (Khosravi-Darani et al., 2013). Here, the calculated cost of PHB production from hydrogen does not consider the treatments costs for the extraction and the separation of the biopolymer from the microbial biomass. This latter consideration is fully justified when PHB are regarded as prebiotic feed additive, able to confer added nutritional value to the produced microbial cells (De Schryver et al., 2010; Defoirdt et al., 2007; Najdegerami et al., 2012).

### 3.2. *The methane and the hydrogen platform: a comparison*

The same range of bio-products (SCP, PHB) can be also obtained by exploiting another type of autotrophic bacteria:

**Table 1 – Hydrogen-oxidizing bacteria yields and estimated cost of microbial by-product (downstream processing costs are excluded).**

| Process/product | Stoichiometric yield (kg product/kg $H_2$-COD) | Higher yield observed (kg product/kg $H_2$-COD) | Final cost[a] (Euro/kg product) | Market cost | |
|---|---|---|---|---|---|
| | | | | Reference product | Cost (Euro/kg) |
| Microbial biomass rich in protein | 0.28 (Ishizaki and Tanaka, 1990) | 0.30 (Ishizaki and Tanaka, 1990; Siegel and Ollis, 1984) | 0.82 | Soymeal | 0.41 (FAO, 2014) |
| PHB | 0.16 (Tanaka et al., 1995) | 0.12 (Volova et al., 2002) | 1.44 | PHB | 1.47 (Lee et al., 1999)[b] |
| $NH_4^+$–N removal and assimilation | 0.03 (Ishizaki and Tanaka, 1990) | – | 7.67[c] | N-removal | 1.0–6.0 (Table 3) |

Note: Other currencies were converted accordingly to the exchange rates of ECB (ECB, 2014).
[a] Calculated on the stoichiometric yield and the chemicals concerned (opex and capex not included).
[b] Possible price of PHB production.
[c] Cost is intended per Kg of nitrogen removed by microbial assimilation.

methane-oxidizing bacteria. These well-studied microorganisms have in the past been implemented in full scale SCP production systems (Nasseri et al., 2011), and tested as protein-rich feed additive for cattle and fish (Øverland et al., 2006; Skrede et al., 1998). In the bio-refinery context outlined in our work, these bacteria might be easily applied by making use of the large amounts of biogas produced at sewage and manure treatment plants. Compared to hydrogen-oxidizing bacteria, methane oxidizing bacteria generally possess a stricter metabolism (i.e. obligate methanotrophy), with lower biomass yields, and similar PHB yields (see Table 2). In addition to the lower biomass yield, they also have lower growth rates and lower protein levels (see Table 2). When compared with hydrogen-oxidizing bacteria, they indeed can be set to work directly on renewable resources such as methane produced from biomass either biological or by gasification. Their downsides are i) lower biomass yield which decreases the maximum volumetric loading rate with a factor 1.5 in a cell-retention configuration or with a factor 10 in flow-through configuration (see Table 2), leading to an increased footprint, and ii) the purity of the produced feed, which is not guaranteed against residual amounts of alkanes (Dalton, 2005). Overall, the application of one type of autotrophic bacteria does not exclude the concomitant use of the other. In view of a hydrogen driven economy, the faster and more efficiently growing $H_2$-oxidizing bacteria are of value in the line of new developments in resource recovery from used water, whereas the already established methane-oxidizing bacteria can represent a technology for upgrading low value methane to microbial biomass used as source of bio-products (Higgins et al., 1981).

## 4. Re-thinking nutrients dissipation: moving towards nitrogen assimilation and upgrade

The conventional nitrification–denitrification process for nitrogen removal from used water is nowadays challenged by new emerging technologies. Advances in research and in biotechnological applications opened a complete new set of processes for nitrogen removal, able to decrease drastically the demand of consumables (chemicals or carbon sources) and energy supply. In the conventional nitrogen removal process, the reduced ammonium nitrogen is first oxidized to nitrate and then reduced to nitrogen gas in the two-step process of nitrification–denitrification. Since anammox (anaerobic ammonium oxidizing) bacteria were discovered, innovative processes able to short circuit the conventional nitrification–denitrification were developed, tested and implemented up to real-scale applications (Kuenen, 2008). Processes based on anammox bacteria are indeed taking over the conventional bioprocess of nitrogen removal, particularly when dealing with high loaded nitrogen water such as sludge reject water (van Dongen et al., 2001), landfill leachate and industrial or agricultural effluents (Van Hulle et al., 2010). For instance, when sludge reject water is recirculated back in the main treatment line, it can account up to 25 percent of the total influent nutrient in the WWTP (Dosta et al., 2007). Therefore, the cost-effective treatment of this

**Table 2 – Comparison between methane-oxidizing bacteria and hydrogen-oxidizing bacteria used in SCP and PHB production.**

| | Methane-oxidizing bacteria | Hydrogen-oxidizing bacteria | Ratio (Hydrogenotrofic/Methanotrofic) |
|---|---|---|---|
| Growth rate ($h^{-1}$) | 0.043 (Yazdian et al., 2005) | 0.420 (Ishizaki and Tanaka, 1990) | 9.8 |
| Cell yield (g CDW/g COD) | 0.19 (Higgins et al., 1981) | 0.28 (Ishizaki and Tanaka, 1990) | 1.5 |
| PHB yield (g PHB/g COD) | 0.14 (Khosravi-Darani et al., 2013) | 0.16 (Tanaka et al., 1995) | 1.1 |
| Protein content (% Protein on CDW) | 60% (Yazdian et al., 2005) | 75% (Volova and Barashkov, 2010) | 1.3 |

highly concentrated side streams is an issue of increasing concern.

Table 3 reports a comparison between the costs for nitrogen removal and recovery using different processes. The biological processes reported include the conventional nitrification–denitrification and the innovative systems based on anammox bacteria. The latter are designed in order to obtain maximum nitrogen dissipation from the effluent (by converting dissolved $NH_4^+$ to $N_2$ gas). The lower integrated cost per unit of nitrogen removed (up to 5 times less expensive, see Table 3) makes anammox-based approaches rather than the thus far implemented (i.e. coupled nitrification and denitrification) the route to go for the treatment of nitrogen-rich side streams such as reject water from sludge treatment plants (Paredes et al., 2007). Nevertheless, even the latter processes are still relying on the assumption that nitrogen has to be removed rather than recovered. Dealing with resource recovery from used water, nutrients are more and more regarded as potential new building blocks. The ammonia can be upgraded to fertilizer rather than be destroyed for fear that it will cause eutrophication (Shu et al., 2006; Wang et al., 2009). The other two processes reported in Table 3 are physical–chemical systems aiming to both nitrogen removal and recovery. The removal of reduced dissolved nitrogen (i.e. ammonium ions $NH_4^+$) by means of stripping is based on the increase of pH and temperature of the effluent prior to removal of ammonia gas with air (Guštin and Marinšek-Logar, 2011). The subsequent reaction of the stripped ammonia with acids allows to recover the nitrogen as e.g. ammonium sulfate $(NH_4)_2SO_4$, which can be used as soil fertilizer (Maurer et al., 2003). Removal of reduced dissolved nitrogen can also be performed together with soluble phosphorus in the so-called MAP (Magnesium ammonium phosphate–struvite) process. When dissolved ammonium ($NH_4^+$) and phosphate ($PO_4^{3-}$) ions are present together with magnesium ions ($Mg^{2+}$) in the molecular ratio of 1:1:1, the precipitation of a crystalline solid allows recovery in form of struvite: $MgNH_4PO_4 \cdot 6H_2O$ (Wang et al., 2009). Amongst these two physical–chemical processes the MAP process generates a more interesting end-product (i.e. struvite), allowing the recovery of both the main nutrients, i.e. nitrogen and phosphate, and moreover of magnesium.

### 4.1. $H_2$-based autotrophic nitrogen assimilation

Moving beyond nitrogen treatment in the form of complete dissipation into $N_2$ gas, $H_2$-oxidizing bacteria might represent an interesting way of recovering this nutrient by converting it directly into valuable microbial biomass. The direct use of hydrogen-oxidizing bacteria in the process of nutrients removal might create an interesting shortcut in the nitrogen cycle. The direct assimilation of reduced ammonia nitrogen by means of hydrogen-oxidizing bacteria would in fact avoid the irrational loop of oxidation-reduction of the already reduced nitrogen. In this case, by establishing a mixed culture of hydrogen-oxidizing bacteria alimented with additionally produced hydrogen (and oxygen), residual ammonium can be removed and converted into microbial biomass, which can be recovered and valorized. The costs of nitrogen removal following the hydrogen shortcut (see Table 1) are in our estimate a factor 2 higher than those of the other well-established biological processes for nitrogen dissipation (i.e. nitrification–denitrification) (see Table 3), nevertheless this re-synthesis approach might be suitable for applications such as upgrading residual ammonium to SCP for aquaculture systems. The latter systems typically suffer of inefficiency in terms of nitrogen input converted into harvestable product (Crab et al., 2007; De Schryver and Verstraete, 2009). Recent studies demonstrated the feasibility and the effectiveness of converting ammonia-nitrogen directly to microbial biomass via heterotrophic microbial metabolism (Ebeling et al., 2006). In this approach, high C/N ratios are set, and the heterotrophically produced microbial biomass is used as additional food source by fish or shrimps (De Schryver and Verstraete, 2009). The microbial biomass produced by the $H_2$-based autotrophic nitrogen assimilation might be used in the same way in aquaculture systems. Moreover, as previously mentioned, the ability of these bacteria to accumulate PHB might enrich the microbial biomass in prebiotic feed additives (De Schryver et al., 2010; Najdegerami et al., 2012).

**Table 3 – Total cost including capex and opex for processes for nitrogen removal and nitrogen recovery.**

| N-removal technique | Type of process | N-recovery | Cost per kg N (Euro) | Reference |
|---|---|---|---|---|
| Nitrification–denitrification | Biological | No | 2.3–4.5 | (Van Dongen et al., 2001) |
| Anammox | Biological | No | 1.0 | (Van Hulle et al., 2010) |
| Air-stripping | Physical–chemical | Yes: $(NH_4)_2SO_4$ | 6.0 | (van Kempen et al., 2001) |
| MAP | Physical–chemical | Yes: $MgNH_4PO_4 \cdot 6H_2O$ | 6.0 | (van Kempen et al., 2001) |

### 4.2. *Nitrogen removal and upgrading in the water factory*

The aforementioned physical–chemical air-stripping and MAP processes might represent the starting line of a $H_2$-based biorefinery integrated in the water treatment plant, thus making it a "water factory". A possible process scheme based on nitrogen removal by air stripping or MAP and subsequent upgrade into added value microbial biomass is proposed in Fig. 2. Nitrogen recovered from anaerobic digestate or reject water might be used as basic nutrient for the build-up of microbial proteins by means of $H_2$-oxidizing bacteria, fed with hydrogen and oxygen produced by renewable-energy-powered water electrolysis. Ultimately, this would connect the production of microbial by-products such as microbial biomass rich in proteins to resource recovery from used industrial or domestic water. Certainly, the use of nitrogen recovered at current costs of 6 Euro/kg N would significantly increase the final cost of the produced biomass, yet these costs relate to total removal of ammonia. Aspects such as land scarcity for conventional crop production and new innovative technologies for recovering the easy to harvest part of ammonia (Desloover et al., 2012; Ulbricht et al., 2013; Xie et al., 2009), certainly offer perspectives in the near future for such microbial route of upgrading nitrogen. The overall process of ammonium removal and reintegration in valuable microbial biomass would include also the major advantage of capturing excess of $CO_2$ from water treatment plant. Either using the $CO_2$ collected from the process of upgrading biogas to bio-methane (Favre et al., 2009; Makaruk et al., 2010; Weiland, 2010), or the $CO_2$ emissions coming from biogas burning for heat and power generation, the implementation of a $H_2$-based biorefinery within water factory can thus decrease its environmental footprint in terms of greenhouse gas (GHG) emissions. Another intriguing aspect of this approach is the high volumetric productivity of the hydrogenotrophic bacteria. Cell concentrations up to more than 90 g CDW/L, with a maximum cell production rate of 5.23 g CDW/L·h and 5.02 g PHB/L·h where already obtained (Tanaka et al., 1995). This offers the possibility to properly design reactor systems that would ultimately give a great advantage in terms of volumetric loading rate or area footprint. The most promising process line to be considered here is the renewable energy-to-hydrogen line, which involves electricity generation from renewable energy sources (wind, solar etc.) and electrolysis of water. This approach offers the major advantage of providing, in a sustainable way, both the hydrogen and the oxygen needed for the microbial biosynthesis by hydrogen-oxidizing bacteria. Together with renewable energy, water electrolysis might be also powered by off-peak electricity, i.e. electrical power available when the energy demand is lower and which would be otherwise wasted. The fact that reforming technical modules are already operational on biogas, converting with high efficiency methane and $CO_2$ to $H_2$ is also of interest. The biogas might then be reformed to valuable hydrogen, with only few traces of CO ($H_2$/CO ratio around 0.97 (Xu et al., 2009)). This process has already been demonstrated during lab-scale tests as able to achieve biogas to hydrogen conversion efficiencies as high as 94–95% (Xu et al., 2010, 2009). Obviously, the key challenge will be the optimal design of the full scale reactor system assuring safety with respect to the used mix of gases (lower explosion limits for oxygen of 6.9% (Tanaka

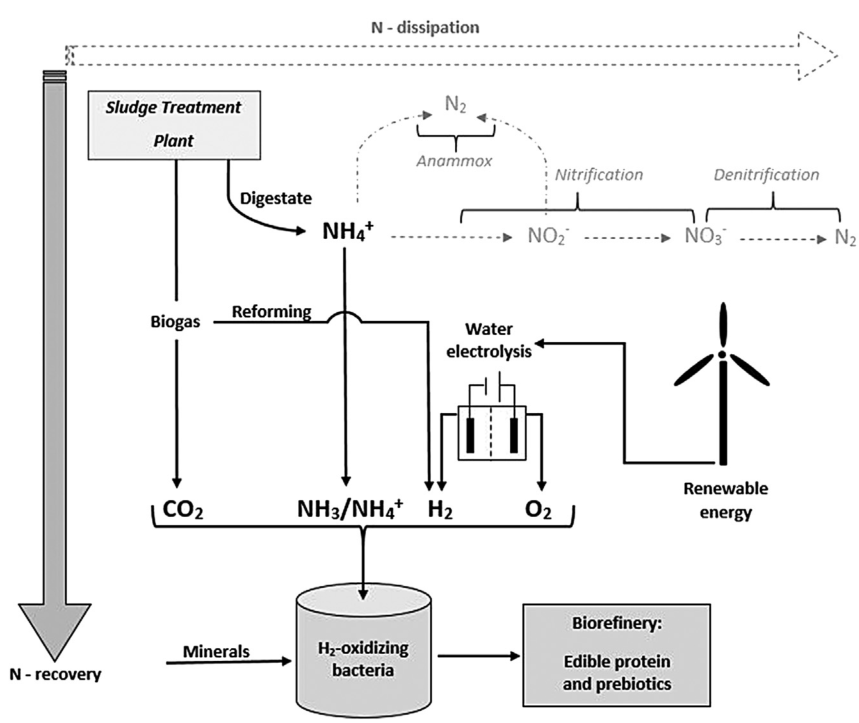


**Fig. 2 – Process scheme of the possible integration of $H_2$-based biorefinery within the used water factory. The gray characters and the dashed lines indicate the conventional approach of nitrogen removal by dissipation into $N_2$ gas.**

et al., 1995)) and the adequate harvesting and processing of the biomass in a way that the latter can be a source of one or more bio-based performance chemicals (SCP, PHB etc.)

## 5. Conclusions and future perspectives

As discussed in the present mini-review, the systematic and rational implementation of hydrogen-oxidizing bacteria, either as axenic cultures, or as evolving microbiomes, offers promising perspectives. Reconsidering the application of these microbial species within a broader and modern framework, matching them with the exploitation of green energies directed to hydrogen production, might give the opportunity to implement innovative process lines in the framework of resource recovery. This might help broadening the possibilities of mitigating the inefficiency and the environmental footprint of conventional used water treatment plants and related resource recovery systems. Powering the bio-refineries with hydrogen can also represents a step forward towards a more integrated and sustainable energy management within the urban context. Currently hydrogen is increasingly regarded as possible energy storage system in the so-called "power-to-gas" approach. There, the inherent instability of renewable energy production (mainly solar and wind energy) and excess of grid electricity (off-peak energy) is mitigated by the production of hydrogen by water electrolysis. The produced hydrogen gas is fed into the gas grid or converted to methane after methanation (Schiebahn et al., 2013). Furthermore, the hydrogen can be used as raw material for chemical, petrochemical, metallurgy and food industry (Winkler-Goldstein and Rastetter, 2013). Such hydrogen production systems can be of use also for upgrading the present WWTP to new "water factories". In this context, hydrogen can serve as electron donor for many metabolic pathways in the broader context of hydrogen-utilizing microorganisms. Processes such as hydrogenotrophic denitrification for tertiary urban wastewater treatment for direct water reuse (Li et al., 2013), as well as hydrogenotrophic sulfate reduction in sulfate-rich industrial wastewater (Esposito et al., 2003) for recovery of valuable heavy metals by the produced biogenic hydrogen sulfide $H_2S$ (Liamleam and Annachhatre, 2007; Papirio et al., 2013), are examples of the broad possibilities offered by this kind of microorganisms in resource recovery. Hydrogen-oxidizing bacteria can, in the approach designed in this paper, be the primary users of such clean and valuable energy carrier. Plenty of technical challenges remain to be dealt with to come to effective upgrading of $CO_2$ and $NH_4^+$−N by means of $H_2$ and $O_2$. Aspects such as gas mass transfer and flammability of the gas mixture (Yu, 2014) can be tackled by employing rational and cost-effective combinations of the latest advances offered by process engineering (Martin and Nerenberg, 2012; Orgill et al., 2013). Moreover, future developments in used water treatment systems will soon provide other possibilities of matching resource recovery with renewable energy production. In this framework, unexpected and intriguing new opportunities are emerging from research on electrochemical and bio-electrochemical systems (BES). Particularly, the generation of hydrogen coupled with the recovery of ammonia from anaerobic digestate (Desloover et al., 2012), reject water (Wu and Modin, 2013) or urine (Kuntke et al., 2014) by means of electrochemical or BES system is a promising line to be followed. It might in fact represent an elegant platform for innovative used water treatment systems coupled to $H_2$-based biorefineries. In view of the increasing demand for quality feed and food protein, the realization of a $H_2$-based biorefinery might lead to the production of high quality feed and food at minimal land requirements. This concept will furthermore mitigate $CO_2$ emission and enhance the sustainability of existing and future water treatment plants. By coupling the conventional disintegrative capabilities of the microbes in general and of the methanogenic microbiome in particular, to a set of novel re-synthesis capabilities of the hydrogenotrophs, the water treatment factory can first generate useful building blocks such as ammonia, carbon dioxide and minerals which can be "accredited for re-use" and effectively upgraded to products desired by the consumer. In this way hydrogen-oxidizing bacteria can re-emerge as pivotal workhorses in processes aiming to the inversion from resource destruction to resource recovery and re-synthesis of valuable products from low value chemical constituents present in various streams of the current bio-economy.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.10.028.

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