APPENDIX 1: NanoRiskCat●●●|●● Template

Literature methodology/sources of information

The following sources of information were used to fill out the NanoRiskCat●●●|●● for C60:

1. Stone V, Hankin S, Aitken R, Aschberger K, Baun A, Christensen F, Fernandes T, Hansen SF, Hartmann NB, Hutchinson G, Johnston H, Micheletti G, Peters S, Ross B, Sokull-Kluettgen B, Stark D, Tran L. 2009. Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES). Available: http://ihcp.jrc.ec.europa.eu/whats-new/enhres-final-report (Accessed July 15, 2010)
2. Shinohara, N., Nakamishi, J., Gamo, M. 2009. Risk Assessment of Manufactured Nanomaterials – C60. Available: http://www.aist-riss.jp/main/modules/product/nano_rad.html?ml_lang=en (Accessed July 15, 2010)
3. Nielsen GD, Roursgaard M, Jensen KA, Poulsen, SS, Larsen ST. In vivo biology and toxicology of fullerenes and their derivatives. Basic Clin Pharmacol Toxicol 2008;103(3):197-208

Human hazard profile

1. **HARN: Does the nanomaterial fulfill the HARN paradigm?**

   Answer: No

   Arguments and explanation: The primary C60 molecule has the shape of a soccer ball and has a diameter of less than 1 nm. At concentrations above the solubilisation limit C60 spontaneously form aggregates or so-called fullerene crystals of 25-500 nm in various suspension including water, ethanol and acetone (Shinohara *et al.* 2009)

2. **Bulk – "Level A CLP": Is the bulk form of the nanomaterial known to cause or may cause serious damaging effects?**

   Answer: Not relevant

   Arguments and explanation: C60 do not have a meaningful bulk parent materials and hence the answer to this question is no by default

3. **Bulk – "Level B CLP": Is the bulk form of the nanomaterial classified for other less adverse effects according to the CLP?**

   Answer: Not relevant

   Arguments and explanation: C60 do not have a meaningful bulk parent materials and hence the answer to this question is no by default

4. **Nano – Acute toxicity: Is the specific nanomaterial known to be acute toxic?**

   Answer: No

   Arguments and explanation: According to Stone *et al.* (2009):*"…different fullerene types have been shown in two studies to have a very low toxicity after oral exposure as no signs of toxicity have been described for the doses tested. From the identified data it might be possible to derive a NOAEL of 2000 mg kg-1 bw for fullerite (mixture of C60 and C70) (Mori et al. 2006) and of 50 mg kg-1 for polyalkylsufonated (water soluble) C60 (Chen et al. 1998b). As only one dose was tested and no dose with an effect has been determined (reported) it might be possible that a higher NOAEL could be determined, especially for the polyalkylsulfonated C60*."…"*Following pulmonary exposure fullerenes have shown no or low ability to induce inflammation or even anti-inflammatory responses.*"… "*The only identified study investigating effects following dermal exposure (human patch test with fullerene soot) found no detrimental outcome.*"**…**"*Following intraperitoneal injection kidney, liver and spleen have been demonstrated to be a target of fullerene toxicity. An LD50 of 600 mg kg-1 was determined. Mice have shown to be able to generate antibodies against the C60 derivatives, which were also active against other nanoparticles (Single-walled carbon nanotubes). The relevance of the findings following intraperitoneal injection for primary routes of exposure (inhalation, dermal and oral) has to be further examined in light of the questionable uptake via these routes.*" (Stone *et al.* 2009).

5. **Are there indications that the nanomaterial causes genotoxic-, mutagenic-, carcinogenic-, respiratory-, cardiovascular, neurotoxic or reproductive effects in humans and/or laboratory animals or has organ-specific accumulation been documented?**

   Answer: Maybe

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Arguments and explanation:

a. **Genotoxicity and mutagenicity:** A number of genotoxicity test have been reported on in the scientific literature. For a recent review, see Stone *et al.* (2009). Studies on C60 suspended in solvents were considered irrelevant for C60 LuBExtreme and so was studies reported on fullerol. A couple of studies has found evidence of genotoxicity of C60. Dhawan *et al.* (2006) investigated whether C60 was able to inflict DNA damage within human lymphocytes, and was detected using the Comet assay, when exposed at concentrations ranging from 0.42 to 2100 µg l-1, for up to 6 hours. Sera *et al.* (1996) investigated the mutagenic effect of fullerene exposure (up to 30 µg per plate, for 48 hours) on Salmonella typhimurium, in light and dark conditions using the Ames test. If exposure occurred within the dark, no mutagenic responses were evident. In contrast, a mutagenic effect was observed, when exposure occurred in the presence of visible light, due to the production of ROS, which interact with DNA to elicit damage, and was typified by the formation of 8-hydroxydeoxyguanosine. Lipid peroxidation was also increased by fullerene exposure in light, further highlighting the oxidative consequences associated with light irradiation. Stone *et al.* (2009) concludes: "Genotoxicity has not been associated with fullerene exposure in a number of studies. Mori *et al.* (2006) investigated the mutagenicity of a C60/C70 mixture. It was illustrated that no mutagenic responses were evident within a variety of Salmonella typhimurium and Escherichia Coli strains, using the Ames test (up to 5000 µg per plate). In addition, within the chromosomal aberration test (in CHL/IU hamster lung cells) no aberrations within the structure or number of chromosomes were apparent. Furthermore, Jacobsen *et al.* (2008) investigated the mutagenicity associated with a number of carbon based nanoparticles, including C60 within the mouse FE1-Muta epithelial cell line. It was demonstrated that C60 exposure (0-200 µg ml-1, 24 or 576 hours) was associated with a slight increase in ROS production in cells and in cell free conditions, but no impact on cell viability was observed. C60 was not capable of eliciting strand breaks, and no alterations in mutation frequency were observed when using the Comet assay." Thus, according to Stone *et al.* (2009) the evidence of genotoxicity of C60 is contradictory and therefore difficult to interpret from the studies conducted so far.

b. Respiratory tract toxicity: Following pulmonary exposure fullerenes have shown no or low ability to induce inflammation or even anti-inflammatory responses according to Nielsen *et al.* 2008 and Stone *et al.* (2009). Sayes *et al.* (2007a),

however, did observe an increase in the percentages/numbers of Bronchoalveolar lavage (BAL)-recovered neutrophils (i.e. white blood cells) after intratracheally instillation of C60 and hydroxylated C60 i.e. C60(OH)24 just 1 day post-exposure. Sayes *et al.* (2007a) also observed a significant increase in lipid peroxidation values and an increase in level of glutathione (GSH), after 1 week. Lai *et al.* (2000) also observed a significant increase in lipid peroxidation products after intravenous administration of 1 mg C60(OH)18 per kg into male mongrel dogs previously induced with infusion/reperfusion injury. In contrast to Sayes *et al.* (2007a), Lai *et al.* (2000) observed a decrease in the GSH level in intestinal tissue. Adelman *et al.* (1994) observed a reduction of the viability of bovine alveolar macrophages compared to control after exposure to sonicated C60 along with increased levels of cytokine mediators of inflammation (i.e. TNF, IL-6 and IL-8) whereas Baierl *et al.* (1996) and Porter *et al.* (2006) found that C60 and raw soot was not toxic towards bovine- and human alveolar macrophages. The alveolar macrophage serves as the first line of cellular defense against respiratory pathogens (Rubins 2003) and hence studies reporting on the effects on alveolar macrophages are of special interests.

c. **Cardiovascular toxicity:** To the best of our knowledge no epidemiological or animal study has been reported on in the scientific literature investigated the effects of C60 on the cardio-vascular system.

d. **Neurotoxicity:** To the best of our knowledge no epidemiological or animal study has been reported on in the scientific literature investigated the neurotoxic potential C60.

e. **Reproductive damage:** Stone *et al.* (2009) recently reviewed the reproductive toxicology of fullerenes. Three studies were reviewed, however only one of them are considered directly relevant for C60 LuBExtreme. In one study C60 had been solubilised in polyvinylpyrrolidone and administered intraperitoneally to pregnant mice (Tsuchiya *et al.* 1996) and in another THF suspended C60 was used to study the cytotoxicity of C60 in Chinese hamster ovary mammalian cell line (Han and Karim 2009). PVP and THF is not used in the production of C60 LuBExtreme and hence these studies were found to be only partially relevant. Collectively, these results, illustrate the potential toxicity of fullerene particles in mammalian ovary cells (Stone *et al.* 2009). However studies are extremely limited in number and in sample size. Only one study identified examined effects on an ovarian cell line model with no studies focused on other organs or

cell types in the female reproductive system. No specific in vitro or in vivo studies were found examining fullerene effects in male reproductive system.

f. **Carcinogenicity:** To the best of our knowledge no epidemiological or animal study has been reported on in the scientific literature investigated the carcinogenic potential C60.

g. Does the nanomaterial accumulate in tissue and/or organs?: According to Stone *et al.* (2009) *"Information regarding the ADME profile of fullerenes is generally lacking, and therefore warrants further investigation in future studies. In the small number of studies described here, it would appear that the majority of fullerenes remain at the deposition site (specifically within the lungs and gut), but that it is also possible for fullerenes to cross cell barriers and to be transported within the blood. Accumulation appears to be predominant within the liver, kidneys and spleen, with evidence of toxicity also manifesting at sites of accumulation. Metabolism of fullerenes has also been suggested, and the consequences of this require consideration. Elimination of fullerenes within the faeces and urine has also been demonstrated, which may reduce their propensity for distribution and toxicity. However, it is relevant to note that the representative nature of the limited number of findings, for all fullerene derivatives is unknown at this time."* Stone *et al.* (2009) furthermore state that: *"The findings from the different studies therefore share the commonality, that subsequent to injection, fullerenes preferentially accumulate within the liver. Therefore it is of high relevance to evaluate the impact of fullerene accumulation on liver function, and to assess the contribution of the liver to the metabolism of fullerenes and, in addition to considering the ability of the liver to facilitate the removal of fullerenes from the body within bile, and therefore the faeces."*

6. **Overall evaluation of human hazard**

We conclude that the color-code that best reflects the human hazard profile of C60 is

● **based on in vitro evidence indicating at least one nanospecific hazard.**

Environment hazard profile

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1. **Bulk – "Level A CLP": Is the bulk form of the nanomaterial classified as CLP Acute 1 or Chronic 1 or Chronic 2?**

   Answer: No

   Arguments and explanation: C60 does not have a meaningful bulk parent materials and hence the answer to this question is no by default.

2. **Nano – LC_{50}<10 mg/l: Is the nanomaterial in question reported to be hazardous to environmental species i.e. LC50 or EC 50 <10 mg/l?**

   Answer: Yes

   Arguments and explanation: According to Stone *et al.* (2009) "*The information available so far leads to the conclusion that non-functionalised C60 is toxic for aquatic organisms. A study with fish observed sub-lethal effects on growth at 0.04 mg l-1*". In the short-term studies with crustaceans lethal concentrations were 7.9 mg l-1 (LC50) for D. magna exposed to sonicated C60 and over 22.5 mg l-1 for copepod species exposed to stirred C60. Long-term exposure of Daphnia magna to 2.5 mg l-1 C60 revealed in a delay of moulting and a significant reduction in offspring. However, the effect on reproduction could have been caused by mortality which occurred from day 5 onwards. A $NOEC_{Daphnia}$ (long-term) should be < 2.5 mg l-1 C60 (Stone *et al.* 2009). Hence non-functionalized C60 has been reported to be hazardous to environmental species i.e. LC50 or EC50 <10 mg/l.

3. **Overall evaluation of environmental hazard**

   We concluded that the color-code that best reflects the environmental hazard profile of C60 is ● based on nanospecific LC50 or EC50 < 10 mg/l.